Characterization of Ovine Pattern Recognition Receptors Expression

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Declaration

I, declare that the content of this thesis submission is my own work and that, to the best of my knowledge and belief, it represents original work and contains no material previously published or written by another person nor material which to any extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where such due acknowledgment has been made in the text.

Signature _____ Date 20.11.2006

Dedication

To the three women in my life; Marjorie, Michelle and Muuluka. You are the shining light in my life and I thank almighty God for that gift. I hope this dissertation is an appropriate tribute for my love for them and for the sacrifices made during the prolonged times we've had to be apart.

To my parents; for showing me the way, instilling the drive and the courage to aspire for greater things.

I extend my deepest gratitude to my wider family (the 'banja'), friends and all those who offered advice, support and guidance during my PhD tenure. Thank you.

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When writing up a PhD it is normally declared that the work is ones own, but the truth is that this work would not have been possible without the contributions of many people within and outside the University of Edinburgh. Practical limitations and space prevent me from listing all of these people specifically; but, in the spirit of great gratitude I say, '*ndalumba kapati*' which in my mother tongue means THANK YOU VERY MUCH in a much deeper sense than its English equivalent.

Abstract

The innate immune system senses pathogens via germ-line encoded proteins called pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs) and C-type lectins. They are able to distinguish a vast range of microbial signatures or pathogen associated molecular patterns (PAMPs) and induce both adaptive and innate, pathogen specific/tailored immune responses. The repertoire of PRRs expressed by each cell type has a bearing on its ability to recognize a specific array of PAMPs on a pathogen and thus mount a specific immune response against such challenge. The cellular content of a tissue thus has a bearing on its ability to mount a competent immune response against pathogens. Age-related differences in immune competence are well documented. Neonates are known to have immature immune systems (and thus increased susceptibility to infections) and postulated to have lower PRR expressions. A discernment of the expression of PRRs in normal cells/tissues enables us understand the significance of these receptors play in immune response development and form a basis for understanding disease pathogenesis. Baseline data also allows deviations from normal to be identified in diseased states. Johne's disease (JD) is a chronic disease of ruminants caused by *Mycobacterium avium paratuberculosis (Map)* and has three clinical forms - asymptomatic, paucibacillary or multibacillary. The innate immune system is known to have a pivotal role in the control of the dissemination in mycobacterial diseases though the precise mechanisms are not well defined. I hypothesized that antigen recognition via PRRs has a definitive role in the development of the different forms of JD.

For part one of this study, blood and tissues were obtained from clinically healthy sheep for RNA extraction. Blood subsets were immuno-stained with specific monoclonal antibodies and DCs into $CD172a^{+/-}$ populations. Definitive populations were obtained using Flow cytometry assisted cell sorting followed by RNA extraction.

To explore PRR expression in foetal immune system, second trimester foetal skins and spleens were collected for PRR mRNA expression determination. Archival, RNAlater® stabilized ileum samples were used to investigate PRR expression profiles of different JD forms.

PRR specific mRNA expression was evaluated using reverse transcriptase quantitative real time PCR.

The spleen, lung and lymph nodes express all TLRs; the kidney expresses high levels of TLR1, 2, 3, 4, 5, 6, but very low levels of TLR8, 9 and 10. The skin expresses low levels of TLR5, 6, 9 and 10 mRNA. CD172a^{+} DCs express most PRRs and MyD88 while CD172a^{-} DCs express TLR2, 6 and MyD88.

Foetal spleens have comparable levels of PRRs except for CD14. Significant differences were observed with TLR1, 4, 5, CD14, CARD15 and Dectin-1 between foetal and adult skin tissues.

Results from the present study show the importance of the following PRRs in ovine Johne's disease discrimination; TLR2, CD14, TLR8, CARD15, dectin-1 and dectin-2. These findings provide an insight into one facet of *Map* innate immune recognition and help to elucidate new target genes for possible mutation analyses and disease genotyping.

List of Abbreviations

Ag	Antigen
APC	Antigen Presenting Cells
bp	base pair
CARD	Caspase Recruitment Domain
cDC	conventional DC
CLR	C-type lectin receptor
CNS	Central nervous system
CR	Complement Receptor
CRD	Carbohydrate recognition domain
DC	Dendritic Cell
dNTPs	Deoxynucleotide triphosphates
ds	double stranded
ECD	Extra-cellular domain
FACS	Fluorescence activated cell sorting
GIT	Gastrointestinal tract
HIV	Human immunodeficiency virus
Hsp	Heat shock protein
ICAM	intracellular adhesion molecule
iDC	immature DC
IEC	intestinal epithelial cell
IFN	interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	IL-1 receptor associated protein kinase
IRF	IFN regulatory factors
JD	Johne's Disease
LaM	lipoarabinomannan
LBP	LPS binding protein
Lnn	lymph node
LPS	Lipopolysaccaride
LRR	Leucine Rich Repeat
mAb	monoclonal antibody
Mal	MyD88 adaptor like
MAPK	mitogen-activated protein kinase
MBP	mannose binding protein
MDA5	melanoma-differentiation-associated gene 5
MHC	Major Histocompatibilty Complex
MIF	Macrophage inhibitory factor
MMR	macrophage mannose receptor
MyD88	Myeloid differentiation primary response gene (factor) 88
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear Factor κB
NK cell	Natural Killer cell

NLR	NOD-LRR receptor *OR* NATCHT-LRR receptor
NOD	Nucleotide-binding Oligomerization Domain
OD	optical density
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral blood mononuclear cells
pDC	plasmacytoid DCs
PGN	peptidoglycan
PKR	Protein kinase R
PRR	Pattern Recognition Receptor
RIG-1	Retinoic acid inducible gene 1
SDHA	succinate dehydrogenase
SIRP	Signal Regulatory Protein
SNP	Single nucleotide polymorphism
SOCS	Suppressor of Cytokine signalling
ss	single stranded
Th	T helper
TIR	Toll/interleukin receptor
TLR	Toll-Like Receptor
TRAF	tumour necrosis factor receptor-activated factor
TRAM	Toll receptor associated molecule
Treg	Regulatory T cells
TRIF	Toll receptor associated activator of interferon

1 Chapter 1 - Introduction

1.1 Host pathogen Interaction

The interaction between a host and an invasive micro-organism is crucial to the outcome of infection. These host-pathogen relationships are characterized by a complex interplay between the host's defence mechanisms and attempts to circumvent these defences by pathogens. Optimal defence against the gamut of micro-organisms such as viruses, bacteria, fungi and parasites, which may invade an animal's body, requires specialized classes of non-specific and antigen specific immune responses. The interactions can range from benign/symbiotic, such as that occurring in the gut to commensals, to fatal competition that may result in the death of the host, the micro-organism or both. These micro-organisms are capable of adapting to, and utilizing elements from, within the host's environment to establish infection and persist. The organisms have developed many methods for ensuring their survival in the host, ranging from evading the host's immune system to down-regulating the immune system. More specific mechanisms used by microbes to avoid host immune responses include:

- Inhibition of antigen presentation (Human cytomegalovirus; (Basta and Bennink, 2003); *Mycobacterium spp* (Berger and Griffin, 2006; Tobian *et al.*, 2003), *Salmonella* (Cheminay *et al.*, 2005)).
- Evasion of host antibodies (Trypanosomes; (Mansfield and Paulnock, 2005)).
- Complement evasion (Vaccinia virus (Kotwal and Moss, 1988)).
- Inhibition, or overproduction, of cytokines (*Toxoplasma gondii*; (Aliberti *et al.*, 2004; Denkers Eric Y. *et al.*, 2003; Denkers, 2003))
- Survival and multiplying in host cells such as phagocytes (Mycobacteria; (Pieters and Gatfield, 2002)) or undergoing latency by persisting in host cells without dividing (Herpes viruses (Novak and Peng, 2005)).
- Phase/antigenic variation (Trypanosomes; (Mansfield and Paulnock, 2005)).

The immunological balance at the interface between a host and its environment is fundamental to the host's well-being. This interface must protect the host from pathogens and also maintain a balance with commensals. It is made up largely of skin, mucosae and cells of the immune system. The cells are divided into sensors and effectors. Dendritic cells (DCs) are the best characterized sensors which also include macrophages and B cells. These cells sense the environment via pattern recognition receptors (PRRs) and PRR engagement causes the cells to respond to environmental changes. PRRs are found on a range of cells and tissues that can respond to and influence the host's response to infection. Upon sensing of pathogens by these cells, immune mechanisms are set into motion that lead to elimination or containment of these pathogens. Paradoxically, hosts also develop mechanisms to ensure the survival of commensal organisms. A fine balance between the effector and regulatory arms of the immune system are required to meet a host's need for pathogen removal and that of tolerance to commensals.

Host defence against pathogenic challenge requires an integrated response from both the innate and adaptive wings of the immune system. Available data adequately demonstrates that Dendritic cells are pivotal in linking the two components of the immune system and determining the shape of the emerging immune response. It has been shown that, upon infection with a pathogen or interaction with an antigen (Ag), antigen presenting cells (APCs) such as DCs and macrophages modulate their PRRs expression. The PRRs bind to pathogen-associated molecular patterns (PAMPs) on the pathogen, and initiate cellular signalling pathways that lead to an immune response via mechanisms such as reactive oxygen and nitrogen intermediates, cytokines, chemokines and co-stimulatory molecules. However, it has also been shown that some organisms have evolved strategies to infect the DCs directly or to evade or usurp their normal function (Humphreys *et al.*, 2006; Jiao *et al.*, 2002). Based on what is known about DCs, the outcome of a pathogenic challenge will be determined by factors such as the identity of DC subpopulations that are engaged, the tissue microenvironment in which activation takes place and the specific signalling molecules involved in the recognition and processing of a particular pathogen or its

product (Kelsall *et al.*, 2002). Pathogen-host interactions will lead to induction of T cell responses that may be more balanced or highly polarized in terms of their Th1/Th2 profiles and this phenomenon is particularly striking with gastrointestinal helminth infestations (Gause *et al.*, 2003; Sher *et al.*, 2003) where there is a strong Th2 response. This polarization may be important for the resolution of certain diseases but may lead to pathologies such as autoimmune disease (Th1) or asthma (Th2). Differences in Th1/Th2 type balance will result from engagement of different DC subsets and this will lead to different immune responses. The importance of this interaction is that it determines the outcome of disease and whether or not micro-organisms are controlled and/or cleared from a host. Differences in the composition of dendritic cell subsets may therefore be associated with resistance and susceptibility to infection with a given pathogen (Moll, 2003).

1.1.1 The immune system

The immune system is tasked with the responsibility of detecting and eliminating invading pathogens. It is able to do this by discriminating between self, non-self and altered self. The mammalian immune system has traditionally been divided into innate immunity and the adaptive immunity. However, it has now come to light that these two wings of the immune system are complexly and tightly interwoven (Degli-Esposti and Smyth, 2005; Vivier and Malissen, 2005) and interdependent. The adaptive immune system depends on the existence of T and B cell antigen receptors, followed by clonal selection and expansion of these receptors with appropriate specificities. This culminates in the development of immunological memory. It takes four to seven days for this process to be fully functional. If this were the only method of combating infection, microbes would overrun the immune system due to this delay. Fortunately, the adaptive immune system does not work in isolation, but in association with the innate immune system that detects the presence and nature of infection, thus providing the first line of defence (Medzhitov, 2001; Reis e Sousa, 2004). Despite its crucial role, the importance of the innate immune system has been overshadowed by the adaptive immune system for over a century (Medzhitov, 2001). It was thought to be a non-specific immune response based on the engulfment and

digestion of microbes and antigen by leucocytes. The discovery and characterization of the mammalian Toll-like receptor (TLR) family and other additional PRRs, together with the important role played by antigen presenting cells, have re-activated interest in the discipline of innate immunity. Innate immunity has also been shown to have considerable specificity, ability to discriminate between self and non-self; to be a prerequisite for the activation of adaptive immunity and to determine the nature of the acquired immune response (McNeela and Mills, 2001). Thus, effective host defence against antigenic challenge and optimal protection depends on a joint response from the two inter-related innate and adaptive wings of the immune system. The innate immune system, being engaged first, is considered to be on top of the hierarchy for the development of immunity. Without the innate immune system, a host is not able to develop a robust and optimal immune response to pathogens.

1.1.2 Innate Immunity.

Greater than 98% of all multicellular organisms lack a functional adaptive immune system. Adaptive immunity is only found in vertebrates (Takeda and Akira, 2003), thus other organisms are entirely dependent on the innate immune system for responding to pathogenic challenge. Similarities between plant and animal innate immune systems have also been demonstrated (Ausubel, 2005; Jones and Takemoto, 2004) and the conserved nature of the innate immune system would thus imply its higher ranking in the immunological hierarchy.

The innate immune system has a mechanical and chemical/cellular component. The mechanical component comprises the epidermis and the mucosae lining the gastrointestinal, respiratory and urogenital tracts that provide a physical barrier to pathogens. The mechanical component includes responses such as cilial movement and peristalsis and also the secretion of antimicrobial substances such as defensins, cathelicidins, secretory leucocyte protease inhibitor (SLPI), lactoferrin and lysozyme (Wira and Fahey, 2004) to aid in pathogen defence. The chemical component comprises a) the cellular constituent which includes mast cells, NK cells, DCs, macrophages and epithelial cells b) pattern recognition molecules and c) proteins or

peptides that hydrolyse pathogens and chemokines (Basset *et al.*, 2003). Early in the study of immunology, the complement system was identified as important in host protection and a key component of the innate immune system. The complement system was originally identified as a heat-labile component in normal plasma that augments or 'complemented' the action of antibodies in the lysis of erythrocytes and bacteria by Bordet and Gengou in 1901.

Thus, broadly, the innate immune system is mediated by phagocytic and cytotoxic cells. In concert, the above lead to the 'first line' of defence to protect a host against pathogenic challenge by destroying or limiting the pathogen and also by alerting the adaptive wing of the immune system.

In light of pathogenic challenge, expression of pattern recognition molecules has recently been shown to increase in cells of the innate immune system and also lead the secretion of antimicrobial substances such as β-defensins (Froy, 2005; Vora *et al.*, 2004; Voss *et al.*, 2006). Paradoxically, defensins have also been shown to activate DCs via TLR4 (Biragyn *et al.*, 2002) which could form a regulatory positive feedback loop. This linking of PRRs of various aspects of innate immunity has strengthened our understanding of the role that PRRs play in overall immunity. Innate immune mechanisms are however limiting and do not result in increased protection over time, or memory. The ability of the innate immune system to mediate induction of adaptive immunity via PRRs enables the subsequent immune response to be specifically tailored to the sensed pathogen.

1.2 Pattern Recognition Receptors

The first step towards the development of an immune response is the recognition of an antigen/pathogen. PRRs are germ-line encoded receptors that act as primary sensors of conserved microbial structures or PAMPs (Janeway, 1992) and PRRs may be cell-surface expressed, located intracellularly, or secreted into tissue fluids. Precise recognition of pathogens is a critical phase in the induction of protective immunity in a host. Accurate recognition determines the initiation, type and extent of

the resulting pathogen-specific adaptive immune response. This activates signalling pathways that lead to the activation of immune and inflammatory mechanisms.

There are three broad groups of PRRs based on cell localization and function:

- secreted molecules that circulate in blood and lymph, such as complement, CD14 and lipopolysaccharide binding protein (LBP);
- surface receptors on phagocytic cells like macrophages that bind the pathogen to facilitate engulfment, such as complement receptors and C-type lectins;
- cell receptors that bind the pathogen initiating a signal leading to the release of effector molecules, such as TLRs.

Recent work has now shown that most PRRs will have two of the above broad characteristics , e.g. C-type lectins are involved in facilitating engulfment, but are also responsible for initiating immune responses. Likewise, a host's reaction to a named pathogen will usually involve PRRs in all these broad groups simultaneously or sequentially.

Pattern recognition receptors also include scavenger receptors, mannose and glycan receptors, Nucleotide-binding Oligomerization Domain proteins (NODs), Protein kinase R (PKR) and complement receptors (CRs) (Basset *et al.*, 2003). PRRs may be expressed on the cell surface or in the intracellular compartment and are not all necessarily cell associated. Mammalian LPS binding protein (LBP), soluble forms of CD14, and mannose binding protein are examples of peripheral sensors, secreted into the blood stream or tissue fluid, that detect host invasion. Soluble PRRs ultimately signal through cell surface receptors (LBP/CD14 via TLR4; MBP via C-type lectins; complement via complement receptors). All PRRs recognize PAMPs displayed by micro-organisms, and their subsequent activation leads to the transcription of appropriate host-defence genes that lead to immune and inflammatory responses aimed at destroying the pathogens. The consequences of PRR engagement therefore include: initiation of phagocytosis (Doyle *et al.*, 2004), activation of pro-inflammatory signalling and the induction of apoptosis (Into *et al.*, 2004; Medzhitov, 2001; Medzhitov and Janeway, Jr., 2002). PRR mediated apoptosis during microbial

challenge is an important aspect of host's defence mechanisms (Haase *et al.*, 2003; Inohara and Nunez, 2003). PRRs also play a role in homeostasis by sensing endogenous ligands and tolerance induction (Gordon, 2002; Rakoff-Nahoum *et al.*, 2004). Rifkin and co-workers (Rifkin *et al.*, 2005) reviewed the role played by TLRs in endogenous ligand recognition and their role in autoimmune disorders. The ability of PRRs to recognise endogenous ligands ('altered self') supports the 'danger theory' of immune initiation as advocated by Matzinger (Matzinger, 1994) in response to dead or dying cells (Shi *et al.*, 2003).

Looking at the increasing number of PRRs discovered in the last decade from the time TLRs were first described, one can only logically extrapolate that what is currently known may only be a small proportion of the overall pathogen sensing receptors in mammalian immunology. This has consequently given rise to more complicated models of pathogen/immune system interaction, antigen recognition and immune regulation mechanisms. As the number of PRRs grows, and the number of endogenous and exogenous ligand increases; together with an expanding role in homeostasis, they may be more appropriately named as *'danger sensors'* to embody their plethora of functions.

1.2.1 The Concept of PAMPs

Pathogen associated molecular patterns are broadly defined as molecular patterns found in pathogens but not in mammalian cells. These include molecules such as peptidoglycan, LPS, flagellin, porins, bacterial DNA and double-stranded RNA. PAMPs tend to be absolutely essential for the survival of a pathogen and are generally evolutionarily conserved in micro-organisms (Barton and Medzhitov, 2003); mutations in most PAMPs are usually not compatible with survival of the pathogen. The term PAMPs is probably a misnomer, as these molecular signatures are also expressed on commensal organisms (Kaisho and Akira, 2004) and altered-self cells (Matzinger, 1994; Matzinger, 2002; Shi *et al.*, 2003). The term 'microbe associated molecular pattern' (Ausubel, 2005), is almost all embodying, and would probably better describe these molecular signatures. It is however, not fully

embodying as, these 'signatures' also include endogenous host molecules termed 'danger signals' such as heat shock protein (Hsp60/70), hyaluronidase, fibrinogen and chromatin-TgG complexes. These 'danger signals' usually indicate injury, tissue damage or cell death (Rock *et al.*, 2005) and pave a way for mechanisms towards elimination of these damaged cells/tissue and tissue repair. Seong and Matzinger (Seong and Matzinger, 2004) proposed that hydrophobicity of molecules is the central trigger that initiates innate immune responses and not PAMPs *per se*. Though controversial, this perspective attempts to unify the still unclear area of the discrimination of common 'PAMPs' found on both pathogens and commensals and how the immune system recognizes altered self molecules and achieves homeostasis.

Three common features make PAMPs ideal targets for innate immune recognition, namely,

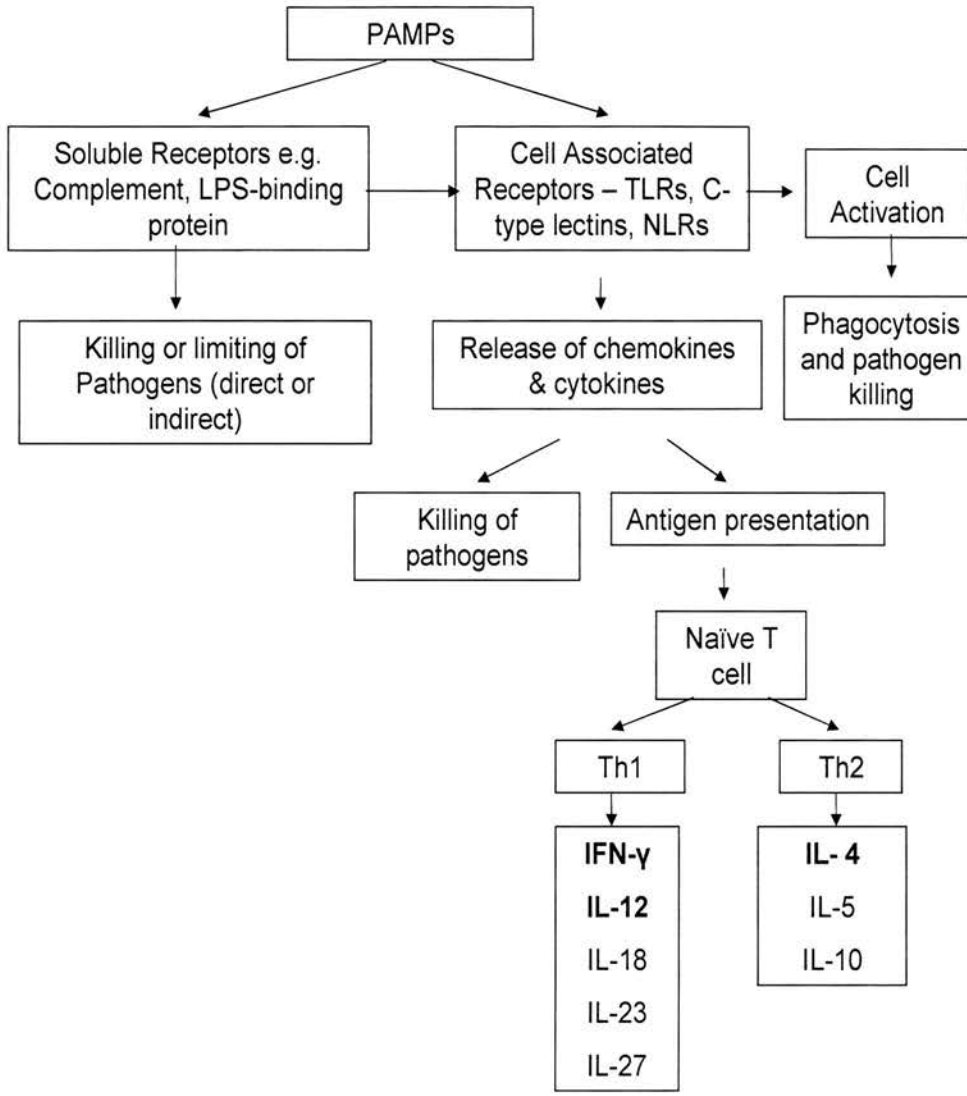
a) They are essential for microbial survival. They are conserved within a class of microbe, since mutations tend to be lethal due to the essential nature of the PAMPs for the microbe's basic physiological functions
b) PAMPs are invariant between organisms of a given class of microbes. Thus, a limited number of pattern recognition receptors can detect the presence of a broad range of microbial infections
c) Host cells do not produce these unique PAMPs, they are only produced by microbes as they perform essential physiological, 'housekeeping' functions. In certain instances, unlike in prokaryotes, these signatures are normally not expressed on the cell surface of host cells but in the cytosol and are thus hidden from immune recognition. However, when cells die by apoptosis, this exposes these signatures to the immune system as 'danger signals'. This enables the innate immune system to distinguish between self and non-self and altered self.

The most widely reported and best-characterized PAMP is lipopolysaccaride (LPS). It is a major component of the outer cell membrane of Gram-negative bacteria and induces a wide range of immune responses in host immune systems via TLR4 and its associated molecules CD14 and MD-2.

A point worthy of noting is that PAMPs are not comparable to virulence factors *per se*. In contrast to virulence factors that developed to ensure the establishment and survival of a pathogen within the hostile environment of a host, PAMPs have evolved to carry out physiological functions. Pathogens produce virulence factors, to among other reasons, facilitate the invasion of host cells, avoid host immune mechanisms or to a change in environmental situations. Thus virulence factors, unlike PAMPs, may vary in different strains of the same pathogen species.

The interaction of PRRs with PAMPs may also result in the activation of the complement (Matsushita and Fujita, 1992) and contact (Kallikrein-kinin) systems, opsonization, induction of co-stimulatory molecule expression and the activation of pro-inflammatory signalling pathways (Hawlisch and Kohl, 2006).

Figure 1.1 Pattern Recognition receptors and their role in immune responses



Schematic illustration of the interaction between Pathogen associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs), both cell-associated and soluble. The resultant cascades lead to pathogen destruction, elimination or limitation. Antigen presentation leads to Th1 or Th2 responses with respective lymphokine profiles.

1.2.2 Toll-like receptors (TLRs)

Toll-like receptors are major cell/cell-surface receptors responsible for initiating immune responses in response to pathogens. TLRs are single-pass trans-membrane proteins characterized by multiple copies of leucine rich repeats (LRR) in the extra cellular domain and a cytoplasmic Toll/IL-1 (interleukin-1) receptor (TIR) homologous domain (Zhang *et al.*, 2004) (see Figure 1.2). TLRs are largely expressed in tissues and cells involved in immune function such as spleen, peripheral blood leucocytes, macrophages, dendritic cells, as well as those tissues that are exposed to the external environment such as the gastrointestinal & urogenital tract, and lung. Akira and co-workers, (Akira *et al.*, 2001), describe TLRs as the most essential signalling receptor molecules from all the PRR that recognize a variety of microbial components resulting in cytokine response of the host. Being essential PRRs involved in PAMP sensing, TLRs have been described as the archetypal PRR. They represent the best-characterized family of PRRs in the evolving field of PRR functional biology. TOLL was originally discovered in the fruit fly, *Drosophilia melanogaster,* larvae as being responsible for dorsal/ventral orientation during embryonic development. It was later found to play an important role in anti-fungal immunity in *Drosophilia* (Lemaitre *et al.*, 1996), where flies that lack TOLL were more susceptible to *Aspergillus* infection. The first mammalian proteins that were structurally related to *Drosophilia* TOLL were discovered in the mid 1990s and called Toll-like receptors (TLRs). Thirteen TLRs have so far been reported in vertebrate species, with ten in humans and thirteen in the mouse, (Takeda *et al.*, 2003; Zhang *et al.*, 2004). TLR1-9 are conserved between humans and mice (Takeda and Akira, 2005) and also in cattle. However, TLR10 is assumed to be functional in humans and cattle but in the mouse the C-terminal half of the gene is substituted to a non-coding sequence suggesting that it may be non-functional. TLR8 is also reported to be non-functional in mice (Sioud, 2006). The TLR11 gene is functional in the mouse, but in the human sequence it has an early stop codon making the human TLR11 gene non-functional. TLRs have not been found to have any conclusive developmental function in humans.

Toll-like receptors have a crucial role in the recognition of 'molecular signatures' of microbial infection, in engaging differential inflammatory signalling pathways, and controlling dendritic cell maturation and differentiation of T helper (Th) cells (Medzhitov, 2001). It is also postulated that TLRs may mediate the inflammatory response that results from damaged host tissues and cells (Beg, 2002; O'Neill, 2004) and are critical mediators of sepsis (Takeuchi and Akira, 2002). TLRs are also able to activate phagocytosis via the co-stimulatory molecules CD80 and CD86 (Doyle *et al.*, 2004).

The different TLRs are distributed differentially between inflammatory cells, recognize different classes of pathogens and induce the production of similar, but not identical, patterns of pro-inflammatory mediators (Akira *et al.*, 2001) (Trinchieri, 2003b) and other specific biological responses. The specificity of TLRs for PAMPs permits them to detect the presence of infection and then drive an appropriate adaptive immune response against that pathogen. TLRs are also observed in other non-immune cells such as adipocytes, cardiac myocytes, intestinal epithelial cells, fibroblasts, platelets and endothelial cells (Akira *et al.*, 2001; Shiraki *et al.*, 2004). The resultant pattern of TLR expression is thus different in different tissues/organs (Werling and Jungi, 2003) and this has a bearing on the specific role played by each TLR in an organ. The differing TLR expression patterns in different tissues has been attributed to the varying complexity of each tissue with regards to somatic and immunological components (Iqbal *et al.*, 2005). Differential expression of PRRs may thus have a bearing on the susceptibility of an organ, individual or species, to either infectious or non-infectious disease (Lehnardt *et al.*, 2003; Wells *et al.*, 2003). To tailor an immune response to suit a particular microbe, multiple TLRs are used to recognize several PAMPs of a microbe simultaneously. The relative input of each individual TLR to an immune response is yet to be quantified since any one pathogen contains numerous ligands specific for different TLRs. There are also differences in TLR activation and signalling between and within host species. Different forms of the same pathogen may also elicit different TLR responses depending on available PAMPs as may the same PAMP on different microbes (Takeuchi *et al.*, 2001).

TLRs may be expressed as homo- or heterodimers. According to many authors, a single TLR is able to bind to such a variety of ligands due to their ability to form heterodimers with other TLRs. This seems to broaden the repertoire of specificities of an individual TLR by forming numerous distinct types of functional heterodimers with other TLRs (Ozinsky *et al.*, 2000) and or PRRs (Gantner *et al.*, 2003; Mukhopadhyay *et al.*, 2004);. However, the full discrimination of pathogenic antigen signals may involve various other adaptor and accessory receptors other than TLRs, which participate in signalling and shaping the eventual immune response. The ability to discriminate between pathogens by using combinations of multiple TLRs has led to the so-called 'bar code' hypothesis (Portnoy, 2005). The stimulation of TLRs by microbial PAMPs leads to downstream signalling cascades that activate both innate and adaptive immunity via antigen presenting cells reliant or independent mechanisms. Thus, when APC through their PRR repertoire read the 'bar code' of PAMPs on a pathogen, they tailor an appropriate immune response.

Table 1.1 The evolvement of the TLR field in immunology

Year	Reported Finding	Reference
1996	Toll found to have a critical role in anti-fungal defence in *Drosophila*	1
1996	First protein structurally related to Toll identified in humans (TLR1)	2
1997	Human TLR4 characterized and its functional role in mammalian immunity determined	3
1998	MyD88 shown to be adaptor molecule for TLR signalling	4
2001	Identification of human TLR10	5

[1] (Lemaitre *et al.*, 1996)
[2] (Taguchi *et al.*, 1996)
[3] (Medzhitov *et al.*, 1997)
[4] (Medzhitov *et al.*, 1998)
[5] (Chuang and Ulevitch, 2001)

1.2.2.1 TLR Structure

Members of the TLR family are characterized by possessing varying number of extracellular LRR domains. The LRR ecto-domain of TLRs is critically responsible for PAMP recognition. It contains 20-25 contiguous copies of the LRR motif. The LRR motif is described as a XLXXLXLXXZ amino acid chain where X represents any amino acid and L is leucine and the Z either a C, S or T (Bell *et al.*, 2003). The complete LRR motif for TLR is **XLXXLXLXXZ***XLXXLXXXXXXXLXX* with the bold portion forming the concave part of the protein and being invariant between LRR subtypes and the italics part forming the outer convex portion of the protein and differing slightly between LRRs subtypes. The cytoplasmic portion of TLRs have a Toll-IL-1 and IL-18 (TIR) homology receptor domain that is responsible for signal transduction, via interaction with adaptor molecules like MyD88, TIRAP, TRAM and TRIF, leading to immune responses. The LRR-ECD and TIR domain are joined by a single transmembrane helix (Martin and Wesche, 2002). However, LRR motifs are also paradoxically present in the virulence factors of pathogenic microbes such as *Listeria monocytogenes, Yersinia pestis,* and *Salmonella typhimurium.* The LRR motif is also found in *Trypanosoma brucei*. The functional implication of the LRR in these pathogens is currently unknown.

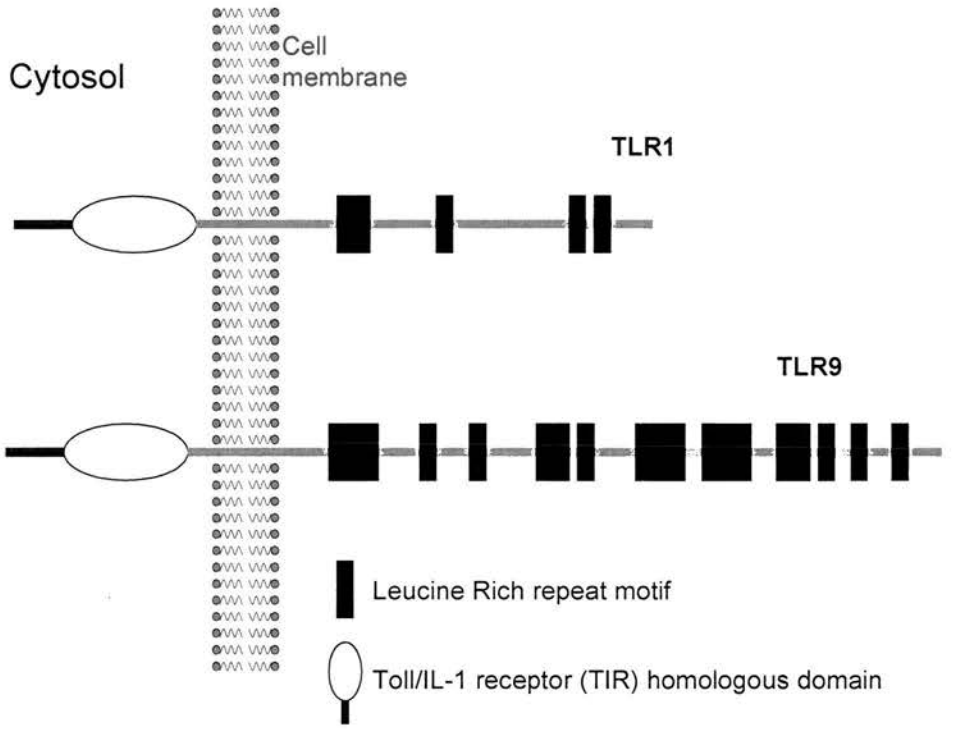


Figure 1.2 Schematic representation of the structure of Toll-like receptors

Schematic representation of the structure of TLRs. (adapted from (Ulevitch *et al.*, 2004). The extracellular domain has varying numbers of LRRs that are responsible for PAMP recognition and ligand binding. The LRRs may also be essential for dimerization. The intracellular domain has a TIR domain that is responsible for signal transduction, via numerous adaptor molecules like MyD88, TIRAP, TRAM and TRIF, leading to biological responses.

1.2.2.2 Toll-like Receptor Expression

TLRs collectively function to alert the immune system of the presence of danger, thus expression is modulated by numerous factors that include pathogen invasion, presence of pathogen products, host tissue destruction, and cytokines. TLRs are expressed on cells involved in the first line of immune defence such as DCs, macrophages, monocytes, neutrophils, endothelial and epithelial cells, B and T cells, Treg, and NK cells. Tissue and cellular distribution also has a bearing on the type of immune response capable of being elicited by a particular tissue at a particular time.

TLR expression can thus be classified as ubiquitous, restricted, or specific. Studies to date have showed that cell surface expression of TLRs is extremely low, ranging from a few hundreds to a few thousand molecules per cell (Janssens and Beyaert, 2003; Visintin *et al.*, 2001) whilst expression of other surface markers like MHC class II will be in the range of a million molecules per cell.

1.2.2.3 Pattern recognition receptor ontogeny

Studies carried out in mice have shown that there is a reduced TLR expression with aging and this has been linked to the decreased resistance to infections in geriatric animals (Renshaw *et al.*, 2002). Neonates are also known to have immature immune systems and are postulated to have a lower level of PRR expressions. This could also be responsible for the poor immuno-reactivity state of the nascent immune system in mammalian neonates. However, mid-gestation foetuses have been shown to be able to mount antigen specific immune responses. Immunization of baboon foetuses has shown that they develop specific antibody responses, independent of the mother (Watts *et al.*, 1999). Thus it is probably a misconception to assume that all mammalian neonates are immunologically naïve. This has been demonstrated in humans by the fact that antigen specific IgE has been demonstrated in cord blood as has tetanus specific IgM in the serum of neonates born from vaccinated pregnant mothers. Marodi (Marodi, 2006) reviews this topic in relation to PRRs and concludes that there are no significant differences in TLR mRNA expression in neonates and adults. Levy and co-workers (Levy *et al.*, 2004) also did not find any difference in basal mRNA expression of TLRs in human neonate and adult monocytes despite an

impaired response to TLR ligands. The Abrahams and co-workers (Abrahams *et al.*, 2004; Abrahams *et al.*, 2005) showed the role TLRs play in early first trimester trophoblasts. It is thus possible that antigen recognition (and thus PRR expression) is fully developed prior to birth but the downstream signalling and/or antigen processing is not. Neonates are specifically known to have a naïve adaptive immune system; they however, must possess a fully functional innate immune system in order to protect themselves against challenges in the antigenically hostile environment into which they are born. Functional species differences have been demonstrated in the level of immaturity in development of the immune system at birth where humans tend to have a more mature immune system than rodents (Durandy, 2003; Renz and Herz, 2002). Neonatal ruminants similarly have a comparatively more developed immune system than mice (Schultz *et al.*, 1973) and could be more developed than humans (Al Salami *et al.*, 1985). Ethical reasons have however hampered the research of many aspects of the ontogeny of the immune system in staged gestation periods in humans.

The immature overall immunity may thus be due to a poor link between the antigenically naïve innate immune system and the adaptive immune system. Specific reasons that have been attributed to this are a) naïve B and T cells that are only able to mount a primary immune response to first time challenge b) defective phagocytosis and c) chemotaxis to infectious sites by immune cells and deficiencies in various aspects of the complement system (Chirico, 2005; Durandy, 2003).

1.2.2.4 TLR Ligands

Most TLRs have had numerous ligands described for them. Some however, such as TLR10 still have no known ligand. Table 1.2 shows examples of known natural and synthetic ligands for the different TLRs.

Table 1.2 Mammalian Toll-like receptors and examples of their ligands

Toll-like Receptor	Natural Ligands (source)	Other Ligands (source)
TLR 1	Tri-acyl lipopeptides (bacteria)	JBT3002
TLR 2	Lipoprotein/lipopeptide (wide variety of pathogens) Lipoarabinomannan (bacterial) Trypanosomal phospholipids (Trypanosomes) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid(Gram-positive bacteria) Porins (bacteria)	JBT3002 Pam3Cys
TLR 3	ds RNA (viruses) Extracellular mRNA (host)	Poly (I:C)
TLR 4	LPS (gram negative bacteria) Fibrinogen, fibronectin (host) β-defensins (host) Heat shock protein 60&70 (host)[6]	
TLR 5	Flagellin (bacteria)	
TLR 6	Di-acyl lipopeptides (mycoplasma)	
TLR 7	ss RNA (viruses)	Imidazaquinoline Bropirimine R-848(Resiquimod)
TLR 8	ss RNA (viruses)	Imidazaquinoline R-848(Resiquimod)
TLR 9	Unmethylated CpG DNA (bacteria) Chromatin-TgG complexes (host)	
TLR 10	*Unknown*	
TLR 11	Profilin from *T. gondii* Uropathogenic bacteria *Escherichia coli*	

[6] Controversy exists, HSP immunostimulatory effect has been ascribed to contaminating LPS (Wallin *et al.*, 2002)

1.2.2.5 Toll-like Receptor Specificity

An increased degree of specificity is conferred onto TLRs by the formation of receptor complexes which are formed between different TLRs or between TLRs and non-TLR moieties (Ozinsky *et al.*, 2000). It is likely that *in vivo*, TLRs act in concert with other PRR and co-receptors, to fine-tune pathogen recognition and eventually drive an appropriate immune response to the pathogen (Armant and Fenton, 2002). The archetypal TLR-TLR heterodimers are the ones formed between TLR2 and TLR1 or TLR6. The TLR2/TLR1 heterodimer recognizes tri-acylated bacterial lipopeptides and mycobacterial lipoproteins whilst TLR2/TLR6 recognizes di-acylated lipopeptides such as those found on *Mycoplasma spp.* (Takeuchi *et al.*, 2001). TLR2 forms a complex with dectin-1 to facilitate the recognition of zymosan and *Candida albicans* (Gantner *et al.*, 2003). TLR4 is known to broaden its ligand range and increase its specificity by forming complexes with CD14 and MD-2 molecules. It is speculated that all TLRs may form heteromeric receptor complexes to enable them to either achieve signalling and/or to increase specificity or diversify ligand recognition potential. Additional molecules that make up these complexes will probably be identified in future as studies of TLR signalling progress. The sequential activation of different TLRs that may have ligands on a particular pathogen has also been proposed as a possible mechanism by which TLRs may tailor an immune response to a pathogen (Weiss *et al.*, 2004). The adaptor molecule(s) combination utilized following ligation of a particular TLR is also proposed to introduce another facet to the specificity of downstream immune response (Horng *et al.*, 2002; Yamamoto *et al.*, 2004).

Thus any pathogen, containing several PAMPS, could be recognized by a combination of different PRRs and accessory molecules on the cells of the host. Each combination of PRR will trigger unique intracellular pathways that lead to an immune response tailored for the invading pathogen. This has been likened to information provided by the different sized lines on a barcode to a scanner.

1.2.2.6 Cellular localization of TLRs

There is a differential spatial distribution of individual TLRs within a cell. This has been demonstrated in mice and humans by the staining of TLRs by mAb. Murine and human studies have shown that TLR1, TLR2, TLR4 and TLR 6 are expressed on the cell surface whilst TLR3, TLR7, TLR8, TLR9 are expressed intra-cellularly (Figure 1.3) in structures such as the endosome/lysosome (Ignacio *et al.*, 2005; Matsumoto *et al.*, 2003). This distribution has a bearing on the function of the TLRs making it more appropriate for the expected kind of natural ligand. The location of each TLR has a critical bearing on the ability of the TLR to access its ligand; thus the nucleic acid recognizing TLRs (TLR3, 7, 8 and 9) are located intracellularly where viruses reside and carry out their replication and in proximity to phagosomes, which may contain apoptotic cells or viral particles. The intracellular location of TLR9 has also been shown to be important in discriminating between self and non-self nucleic acids (Barton *et al.*, 2006). TLRs are also able to migrate from the cytosol to the surface (Xu *et al.*, 2005) and vice versa (Husebye *et al.*, 2006). The exact mechanisms and purpose for the induction of this migration are currently unknown, though Husebye and co-workers (2006) propose that TLR4 moving into the endosome is a regulatory mechanism that is used to terminate TLR signalling.

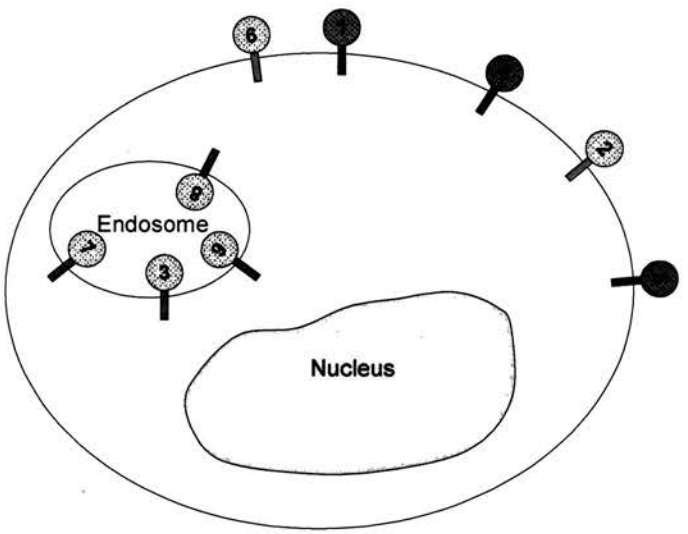


Figure 1.3 Cellular localization of TLRs

Schematic representation of TLR cellular localization. TLRs 3, 7, 8, and 9 are expressed intracellularly in the endosome whilst all the other TLRs are expressed on the cell surface. This may have a functional impact on what type of ligands these TLRs may be able to recognize. Thus, depending on the cellular location, the TLR ectodomain face the cell wall's exterior or interior, for cell surface and intracellular TLRs respectively.

1.2.2.7 TLR Signalling Pathways

TLRs activate signalling pathways that are critical for the induction of immune responses to various microbes. Presently, there are four known adaptor molecules through which TLRs are able to achieve intracellular signalling. These molecules are myeloid differentiation primary response gene (factor) 88 (MyD88), Mal (MyD88 adaptor like - also known as TIRAP)[7] (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001), Toll receptor associated molecule (TRAM) (Yamamoto *et al.*, 2003), and Toll receptor associated activator of interferon (TRIF) (Yamamoto *et al.*, 2004). All TLRs other than TLR3 activate a common signalling pathway that includes MyD88, Interleukin 1 receptor (IL-1R)-associated protein kinase (IRAK) and tumour necrosis factor receptor-activated factor 6 (TRAF6). This TLR ligation eventually culminates in the activation of nuclear factor-κB (NF-κB) transcription factors, as well as mitogen-activated protein kinases (MAPKs) (Yamamoto *et al.*, 2004) extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) (Barton and Medzhitov, 2003) leading to a downstream immune response. However, it has been demonstrated that the signalling has at least two distinct pathways - a MyD88 dependent one that leads to the production of pro-inflammatory cytokines with rapid activation of NF-κB and MAPK; and a MyD88-independent one associated with the stimulation of interferon beta and maturation of dendritic cells with slow activation of NF-κB and MAPK (Yamamoto *et al.*, 2004). This activation leads to the induction of various genes that function in host defence, including pro-inflammatory cytokines (IL-12 and TNF-α), chemokines, major histocompatability complex (MHC) and co-stimulatory and adhesion molecules. This activation may also lead to an increased antigen presentation capacity in APCs. Other multiple effector molecules that directly destroy microbes such as inducible nitric oxide synthase and anti-microbial peptides are also induced. TLR2 signalling via MyD88 has also been shown to induce apoptosis via the Fas associated death domain molecule and caspase 8 (Aliprantis *et al.*, 2000). TLR3 activation signals exclusively via TRIF, although it

[7]Yamamoto and colleagues (Yamamoto *et al.*, 2004) concluded that TIRAP is an adaptor molecule but not responsible for MyD88-independent pathway. It is necessary for aspects of the TLR2 & 4 MyD88-dependent signalling.

was earlier thought to also signal via MyD88. This has been ascribed to the fact that TLR3 lacks a conserved proline residue found at the cytoplasmic end of other TLRs (the BB loop) that is necessary for the recruitment of MyD88 (Xu *et al.*, 2000) but not needed for signalling via TRIF. Nagai and co-workers (Nagai *et al.*, 2006), also showed that TLR signalling has an effect on haematopoiesis by driving stem cells towards myelopoeisis. They also proposed that via MyD88, this may be a potential route for immune cell replenishment during infection and subsequent inflammation. Thus, the conclusion is that each TLR signalling has an overlapping, albeit distinct function that is largely dependent on intracellular molecules (Kaisho and Akira, 2004). MyD88 is however, the principal adaptor molecule required for most TLR signalling, down-stream immune responses and modulation. This is aptly demonstrated by MyD88 deficient mice that have been shown to be unable to drive Th1 responses but whose Th2 responses remain intact (Goldstein, 2004) and they succumb easily to bacterial infections. MyD88 is also involved in IFN-γ induced cytokine and chemokine modulation (Sun and Ding, 2006) and IL-1 mediated NF-κB activation (Wesche *et al.*, 1997).

The complexity of TLR signalling is summarized schematically in Figure 1.4. This is by no means the complete signalling pathways as new aspects to TLR signalling are still being discovered. Recently, Oda and Kitano (Oda and Kitano, 2006) comprehensively reviewed the complex signalling pathways of TLRs.

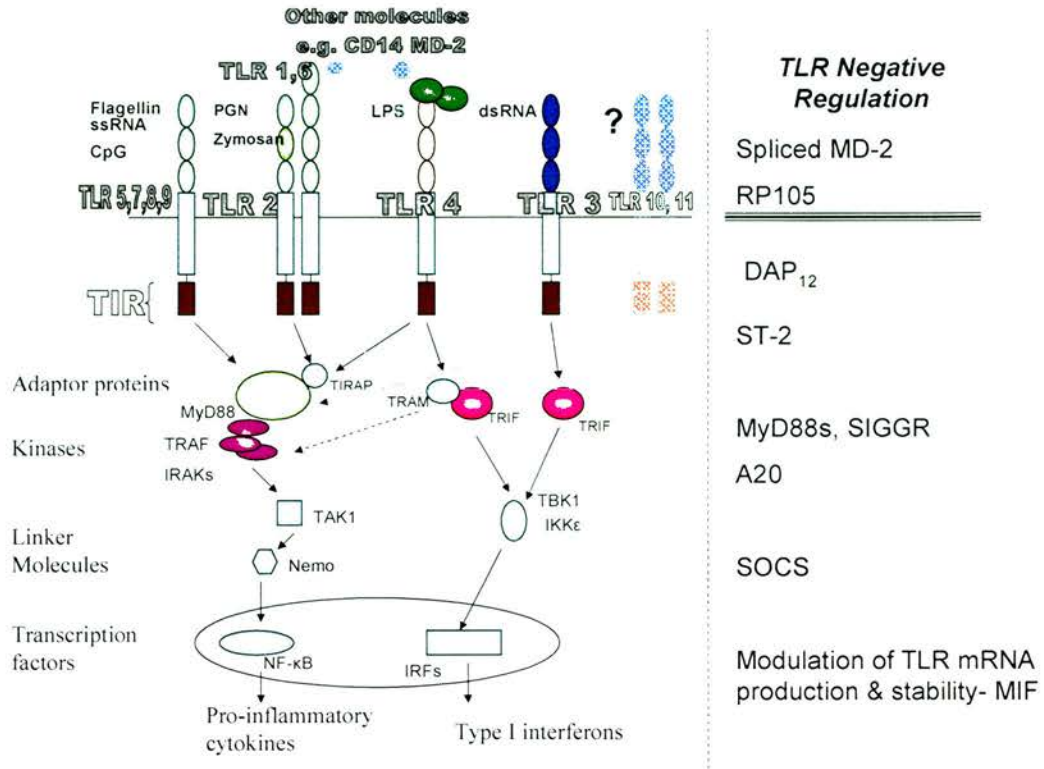


Figure 1.4 Toll-like receptor signalling

Schematic presentation of Toll-like receptor signalling and molecules known to regulate signalling. Interaction between the TLR extracellular LRR domain and its specific ligand leads to the interaction between its intracellular TIR domain and that of adaptor molecules such as MyD88/TIRAP (All TLRs except TLR3) and TRIF (TLR3 exclusively and possibly TLR4 - there is conflicting data on TLR4 in literature). TLR5 signals via MyD88 only without TIRAP. The MyD88 pathway leads to activation of an intracellular cascade involving TRAF6, IRAK-1/4 and the transcription factor NF-κB that results in pro-inflammatory cytokines. The TRIF pathway results in type I interferons via activation of the transcription factors IFN regulatory factors (IRFs) 3 and 7. Numerous molecules regulate TLR signalling at different levels. Spliced MD-2 and RP105 (a TLR4 homologue) interfere with ligand binding at the LRR ECD and thus regulate TLR signalling by competing for binding sites and ligand respectively. The same applies for spliced MyD88 (MyD88s) in the cytosol.

1.2.2.8 Negative regulation of TLR signalling

Downstream effector immune responses generated by TLR ligation are balanced by numerous regulatory molecules and pathways in order to maintain immune homeostasis. Otherwise pro-inflammatory cytokines produced following TLR ligation, when produced in excess would lead to detrimental effects to the host. An excessive and inappropriate immune response would lead to a severe hyper-inflammatory disorder such as inflammatory bowel disease, autoimmune syndromes (Karin *et al.*, 2006), or severe sepsis. Numerous molecules have recently been identified that have a regulatory effect on TLR signalling and include Suppressor of Cytokine Signalling SOCS (Baetz *et al.*, 2004; Mansell *et al.*, 2006), Single Ig-domain containing IL-1R related (SIGIRR) (Qin *et al.*, 2005; Wald *et al.*, 2003), ST2 (Akira and Takeda, 2004; Kobayashi *et al.*, 2005; Kobayashi and Flavell, 2004; Kobayashi *et al.*, 2006), IRAK-M (Kobayashi *et al.*, 2002), spliced variants of MyD88 (MyD88s) (Burns *et al.*, 2003; Janssens *et al.*, 2003) and MD-2(sMD-2), A20 (Gon *et al.*, 2004; Silverman and Fitzgerald, 2004), Tollip (Zhang and Ghosh, 2002), MIF, DAP_{12} (Hamerman *et al.*, 2005), Triad3A (Chuang and Ulevitch, 2004) and the TLR4 homologue RP105 and its accessory molecule MD-1 (Divanovic *et al.*, 2005). These molecules achieve immune homeostasis, by acting at different levels of the TLR signalling pathway (Figure 1.4), to modulate the production of pro-inflammatory cytokines following antigenic challenge and inflammation. The exact mechanisms by which most of these molecules regulate TLRs are not fully understood, but are becoming clearer as TLR signalling is mechanistically dissected by various researchers. The spliced variant of MyD88, MyD88s, functions as a negative regulator by competitively preventing the recruitment of IRAK-4 to the TLR/MyD88 complex. RP105 and its accessory molecule MD-1 were thought to act as a decoy receptor complex by taking up most of the available ligand. However, since RP105 has no signalling domain it was thought to act as a 'molecular sink' by reducing the effective ligand interaction with TLR4 for intracellular signalling. Recent evidence however, shows that the RP105/MD-1 complex physically associates with the TLR4/MD-2 receptor complex making it unable to bind LPS.

1.2.2.9 An overview of the different TLRs

TLRs induce and/or suppress the transcription of greater than 2000 host defence genes (Aderem, 2003) that include cytokines, chemokines, co-stimulatory molecules, antimicrobial peptides and MHC molecules. Consequently, TLR stimulation triggers increased antigen presentation capability and DC maturation and down-stream immune responses. TLRs have also generated interest due to their therapeutic potential especially in tumour medicine (Lawton and Ghosh, 2003; Ulevitch, 2004) and also in vaccine development (van Duin *et al.*, 2006).

Toll-like Receptor One (TLR1), Two (TLR2) and Six (TLR6) are classified into one family due to their sequence homology and ability to form functional heterodimers. The TLR2 gene has the highest number of known natural ligands described (Wetzler, 2003). This promiscuity has led TLR2 to be a quite widely studied TLR. Unlike other TLRs, it is known to further its recognition spectrum and specificity by forming functional heterodimers with the related TLRs 1 and 6. TLR2 signalling has recently and consistently been shown to bias towards Th2 type immune responses (Dillon *et al.*, 2004; Redecke *et al.*, 2004) and suppresses Th1 via various mechanisms including self Hsp (Zanin-Zhorov *et al.*, 2006) and Tregs. A functional soluble form of TLR2 has also been described (LeBouder *et al.*, 2003). This family of TLRs signals exclusively via the MyD88 adaptor molecule coupled with the accessory molecule TIRAP.

The TLR3, 7, 8 and 9 superfamily recognizes nucleic acids. TLR3, 7 8 and 9 are also unique in that they are not expressed on the cell surface but rather intracellularly in endosomes (see Figure 1.3). The members of this family generally recognize viral and bacterial nucleic acids although recent findings implicate them in the recognition of self nucleic acids during tissue injury and apoptosis (Sioud, 2006). Species differences in protein functionality also exist; TLR8 is purported to be non-functional in mice but functional in humans. Other than TLR3 that signals exclusively via TRIF, the others members of this superfamily are known to signal only via MyD88.

TLR4 was the first mammalian TLR demonstrated to function as a PRR (Medzhitov *et al.*, 1997) and together with TLR2 has been a widely studied TLR. TLR4 was

shown to be a major receptor for LPS but it was quickly shown not to be the sole receptor involved in LPS recognition. The complex recognition has been shown to involve LBP, CD14 and MD-2. TLR4 signals via MyD88/TIRAP and also in a MyD88-independent manner via TRIF/TRAM.

TLR5 is expressed on epithelial cells, macrophages/monocytes, CD4+ T cells and DCs. TLR5 recognizes extracellular bacterial flagellin of both Gram negative and Gram positive bacteria. Flagellin is a protein ligand of TLR5 however the specific TLR5 binding site on the flagellin has not been determined. TLR5-independent recognition of flagellin has been described to take place in the cystosol by Ipaf, a member of the NLR family (Franchi *et al.*, 2006). TLR5 signals exclusively via MyD88.

Toll like receptor ten is an orphan TLR although it is closely related to TLR1 and TLR6. No ligand has yet been identified for TLR10 but it is expressed preferentially on B cells and DCs (Hasan *et al.*, 2005). Species differences in the functionality of TLR10 exist; it is functional in cattle and humans but not in mice. Research on TLR10 has thus been particularly hampered by the lack of a functional receptor in mice. It is also known to be abundantly expressed in immune-cell rich tissues such as lymphnodes and the spleen (Chuang and Ulevitch, 2001). Human TLR10 has recently been shown to form heterodimers with TLR1 and TLR2 (Hasan *et al.*, 2005). The functional implication of these heterodimers is however still unclear.

Other Toll-like receptors such as TLR11 and TLR13 have been described in the mouse but no functional equivalents have been found in humans. TLR11 is functional in mice and is a receptor for uropathogenic bacteria and the ligand profilin found on *T. gondii*.

1.2.3 TLR-independent recognition of pathogens

TLRs have dominated the field of PRRs but are by no means the only mechanisms by which the innate immune system recognizes pathogens. The innate immune system is endowed with many other means of recognizing antigen that include the complement system. TLRs are the most widely researched PRRs, but there are an increasing number of other PRRs that have been reported. In the recent past, numerous TLR-independent signalling pathways have also been well characterised as being involved in innate immune recognition. Such non-TLR PRR proteins include NLRs, C-type lectins (Brown, 2006), Retinoic acid inducible gene 1 (RIG-1) (Bird, 2005) and melanoma-differentiation-associated gene 5 (MDA5) (Kato *et al.*, 2005; Kato *et al.*, 2006). However, non-TLR pattern recognition receptors have generally been less appreciated in the earlier part of the last decade despite the initial PRRs discovered such as complement and complement receptors, CD14 and lectins being non TLRs. This has however become a rapidly growing area of PRR research as it has become evident that these PRRs are also critical in initiating and/or modulating innate immune responses. Non-TLR PRRs may induce intracellular signalling that leads directly to inflammatory responses or may work together with TLRs by presenting PAMPs to TLRs (such as CD14 and TLR4). There is growing evidence to suggest signalling interplay and overlap between TLRs and non-TLR PRRs such as NLRs (Creagh and O'Neill, 2006). MDA5 and RIG-1 are CARD domain containing, cytoplasmic RNA helicases that recognize double stranded RNA. Through their helicase domain, they are able to sense the dsRNA of viral origin independent of TLR3 and induce type I IFN responses using the CARD domain signalling pathway. Table 1.3 shows some examples of non-TLR PRRs.

Members	Broad Category	Location	PAMP	Organism
NOD1, NOD2, NALP3	NLR (CATERPILLER)	Cytoplasmic	GM-Tri_{DAP} MDP	*Shigella* *Mycobacteria*
C1q, C3	Complement	tissue fluid		Microbial surface, HIV
CR3, CR4	Integrins	Membrane bound	Mannose, β-glucan	Complement bound microbes, LPS
Dectin1, Dectin2	C-Type Lectins (non-classical)	Membrane bound	β-glucan Unknown	*Candida spp*
DC-SIGN L-SIGN, SIGNR1 SIGNR3	C-Type Lectins (classical)	Membrane bound	gp120	HIV
CD14	LRR	Cell surface, tissue fluid	LPS, PGN, LaM	*E. coli; Mycobacteria*
RIG-1 MDA5	Helicase proteins with CARD domain	cytoplasmic	ds RNA	RNA viruses

Table 1.3 Non-TLR pattern recognition receptors

Table showing examples of non-TLR PRR and the broad category to which they are derived (adapted from (Brown, 2006). The cellular location and main ligands described together with organisms possessing such PAMPs or known to activate the family of receptors.

1.2.4 Non-TLR Pattern Recognition Receptors

1.2.4.1 Caspase Recruitment Domain 15 (CARD15)/ Nucleotide-binding Oligomerization Domain 2 (NOD 2)

CARD15/NOD2 is a member of the NOD-leucine-rich-repeat (LRR) protein family (also called NATCHT-LRR (NLRs) or CATTERPILLER family) (Ting and Davis, 2005). Martinon and Tschopp ((Martinon and Tschopp, 2005; Tschopp *et al.*, 2003) review this family of PRRs, members of which are defined as sharing a tripartite domain structure which has a C-terminal peptide recognition (LRR) domain), a central NOD domain and an N terminus made up of protein-protein interaction domains such as CARDs or a pyrin domain (Figure 1.5). At the time of its discovery, the presence of LRR suggested that, like TLRs, it could be involved in the innate immune recognition of PAMPs. The mammalian CARD15 gene has structural homology to plant disease resistance genes (R) (Jones and Takemoto, 2004). R genes are known to be important in recognition of and defence against various intracellular pathogens in plants which do not possess an adaptive immune system (Ausubel, 2005;Jones and Takemoto, 2004). CARD15 is expressed intracellularly in monocytes (Berrebi *et al.*, 2003), and antigen presenting cells, and allows these cells to recognize muramyl dipeptide (MDP), a metabolite of bacterial peptidoglycan (PGN). CARD15 signalling leads to the enhancement of apoptosis and/or the activation of NFκB via the CARD domains interacting with RIP2 (Inohara and Nunez, 2001; Opitz *et al.*, 2004). The RIP2 interacts with I-kappa B kinase (IKKγ), the regulatory unit of the IKK complex, culminating in IkBα phosphorylation and degradation. The activated NFκB translocates into the nucleus where it mediates pro-inflammatory gene transcription (Strober *et al.*, 2006) and release of anti-microbial defensins (Voss *et al.*, 2006). In addition to the NFκB pathway, CARD15 recruitment of RICK may also activate MAPKs, p38, Erk, and Jnk (Kobayashi *et al.*, 2005).

The most extensive research area of the CARD15 gene has been its function in bacterial sensing in the gastrointestinal tract (GIT) and on intestinal cells, but it has also been implicated in directly having anti-bacterial properties (Hisamatsu *et al.*, 2003). Lack of these anti-bacterial properties in mutant CARD15 has thus been advanced as the reason for its association in Crohn's disease.

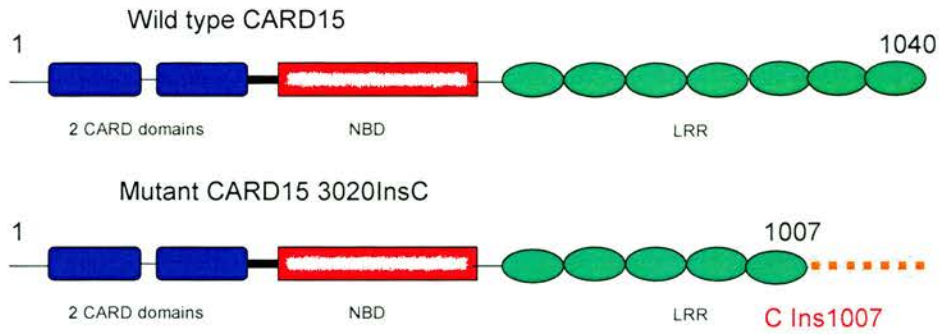


Figure 1.5 The structure of normal type and a mutant CARD15.

Schematic representation of normal and mutant CARD15 **Top**: Schematic representation of the structure of normal human CARD15 gene showing the LRR region that is responsible for ligand recognition and the two CARD domains that take part in intracellular signalling. These two are separated by a nucleotide binding domain. Total length is 1040 amino acids. **Bottom**: Structure of mutant human CARD15 gene showing the truncated LRR region that is incapable of ligand recognition and intracellular signalling. Total length of mutant is 1007 amino acids. Other NLRs would have amino-terminal pyrin domains instead of CARD domains for intracellular signalling.

Counter-intuitively, CARD15 signalling also seems to negatively regulate TLR2 intracellular signalling (Figure 1.7). Using CARD15 deficient mice ($CARD15^{-/-}$), (Watanabe *et al.*, 2004) demonstrated that CARD15 signalling inhibits TLR2 Th1 response by regulating NF-κB signalling at the c-Rel subunit. Thus, CARD15 deficiencies tend to increase TLR2 mediated Th1 responses. TLR2 is normally located on the extra-cellular aspect of immune cells where it senses peptidoglycan (PGN). During antigen uptake, PGN may be incorporated into the cytosol of APCs, where it is broken down by intracellular hydrolases into muramyl dipeptide (MDP) which is in turn detected by CARD15. This state of affairs is usually in a *modus vivendi* and coordinates the response of APC and subsequently the immune system to pathogens presenting with PGN.

CARD15 expression is also regulated by proinflammatory cytokines such as TNFα and IFNγ (Iwanaga *et al.*, 2003; Rosenstiel *et al.*, 2003) and this has a bearing on the fine-tuning of the over all immune response mediated by CARD15 and this may be a

regulatory mechanism or favour a hyper-inflammatory state development. Figure 1.7 outlines the latest proposed mechanism of CARD15 and its implication for the CARD15 mutations which may explain Crohn's disease (and other auto immune granulomatous diseases such as Blau syndrome). This theory ties in with the bacterial basis of these autoimmune diseases, although it may not be accepted by some researchers who propose that these autoimmune diseases have no bacterial cause.

1.2.4.2 CD14

CD14 is a 48/56 kDa glycosylphosphatidylinositol (GPI) anchored glycoprotein with multiple LRRs. It is mainly present on the surface of myeloid cells, but also on B cells and some other non-myeloid cells (Antal, 2000). CD14 was first described as a receptor for lipopolysaccaride and other bacterial cell-wall components in the early 1990s. CD14 was initially described to typically recognize the lipid A portion of LPS. It was purported to be expressed largely or exclusively on myeloid cells. Recently, CD14 expression has been ascribed to cells other than those of myeloid origin and has been reviewed by Jersmann (Jersmann, 2005).

Other than this cell membrane-bound form (mCD14), CD14 is also present in a circulating soluble form (sCD14) in serum. This soluble free-form arises from cleavage of the GPI anchor of the membrane bound CD14. sCD14 has been postulated to be a co-stimulator molecule in modulating PRR-mediated immune responses in cells that do not express mCD14. In the last few years it has generally been accepted that CD14 is a PRR for many ligands including LPS of Gram negative bacteria, lipoarabinomannan of mycobacteria, apoptotic cells, whole bacteria, microbial cell wall components, bacterial products, PGN and lipotechoic acid. However, CD14 lacks a trans-membrane domain. This shortcoming has led to the proposition that it cannot carry out intracellular signalling to achieve an immune response on its own but there is a need for a co-receptor or accessory molecule to achieve downstream immune signalling. The cooperation between CD14 and TLR4, MD-2 and with TLR2 are well described, and this could explain the ligand range that has been hitherto ascribed to CD14. Recently, the synergistic association of CD14 with enhanced TLR3 signalling has been reported (Lee *et al.*, 2006).

1.2.4.3 C-Type Lectins

The calcium-dependent, C-type lectin receptors (CLRs) are a large group of receptors with carbohydrate recognition domains (CRD) that have a high affinity for a variety of sugars and (to a lesser extent) some proteins. They are central to various physiological processes that include enzyme trafficking, immunity, tissue homing, and wound repair (Meyer-Wentrup *et al.*, 2005). The main function of CLRs in antigen presenting cells is to facilitate antigen uptake and degradation to augment further Ag processing and presentation to B and T cells in the context of MHC. C-type lectins bind to specific carbohydrate antigens present on the surface of pathogens (e.g. *Candida albicans*, HIV and *Mycobacterium tuberculosis*) or constituents of secreted products of pathogens (Gordon, 2002). They are also postulated to be involved in the regulation of homeostatic balance and/or tolerance via the recognition of glycosylated self antigen. *Mycobacterium tuberculosis* is known to secrete glycosylated antigen targeting C-type lectins that leads to immunosuppression of host cells. C-type lectins show great heterogeneity in mRNA and have a tendency to be alternatively spliced into multiple isoforms.

The members of the C-type lectin family are numerous and include; DEC-205 (CD205), Mannose receptor (CD206), Langerin (CD207), DC-SIGN (CD209), Dectin-1, L-SIGN and SIGNR1.

1.2.4.3.1 C-Type Lectin signalling

CLRs possess cytoplasmic signalling motifs such as immunoreceptor tyrosine-based activation motif (ITAM) (Humphrey *et al.*, 2005) or immunoreceptor tyrosine-based inhibition motif (ITIM) that they use for downstream immune signalling. (Meyer-Wentrup *et al.*, 2005). Signalling is via the recruitment of a tyrosine kinase Syk, which leads to cytokine production (Rogers *et al.*, 2005). Syk-independent signalling mechanisms are used by C-type lectin mediated phagocytic uptake (Herre *et al.*, 2004b) in macrophages.

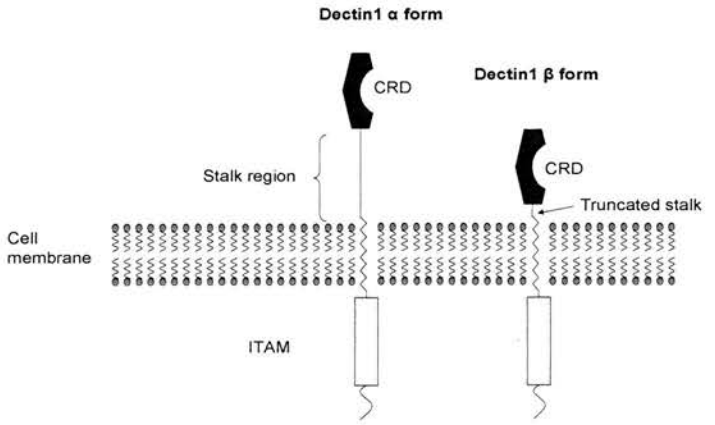


Figure 1.6 Schematic representation of dectin structural architecture

Schematic representation of dectin receptor and the alternatively spliced variant. The normal α form (left) with a long extracellular stalk and the β form (right) with a short stalk. Both forms have a similar extracellular CRD that recognizes β-glucans and a hitherto unknown ligand on T cells. The intracellular N terminal contains the ITAM domain that is used for signaling.

1.2.4.3.2 Dectin-1 (C-type lectin domain family 7, member A – CLEC7A)

Dectin1 is a type II surface lectin which was initially thought to be expressed exclusively or preferentially on dendritic cells but was later reported to be expressed on many more cell types and tissues (Brown and Gordon, 2001). The full physiological function of dectin-1 is still rather unclear. Dectin-1 is known to bind to β-glucans (Herre *et al.*, 2004a) and also a ligand on T cells that is yet to be identified (Ariizumi *et al.*, 2000). Recently, curdlan has been shown to be a selective dectin-1 agonist that results in NFκB induction via SyK and activation of DC maturation. Due to the great heterogeneity in mRNA shown by C-type lectins, different isoforms of dectin-1 are present human, mouse and cattle (Heinsbroek *et al.*, 2006; Willcocks *et al.*, 2006). The well-known spliced variant of dectin-1 is the β-isoform with a truncated extra-cellular stalk (Figure 1.6) but an intact functional CRD. The isoforms may have different functions as they seem to have cell and strain-specific expression in humans and mice (Heinsbroek *et al.*, 2006), respectively. With murine dectin-1 (β-isoform), functional deficits in binding zymosan were seen at temperatures lower

than 37°C but at body temperature, functional binding was similar between the two isoforms (Heinsbroek *et al.*, 2006). After binding of dectin-1 with a specific ligand, the different intracellular signalling can facilitate the uptake of an antigen by phagocytosis (Herre *et al.*, 2004b) and receptor mediated endocytosis and also leads to the induction of reactive oxygen production. In collaboration with TLR2 and TLR6, dectin-1 can also lead to higher specificity immune recognition and responses (Gantner *et al.*, 2003; Netea *et al.*, 2006) and the TLR2 dectin-1 cooperation may lead to immune tolerance (Dillon *et al.*, 2006). Rogers and co-workers (Rogers *et al.*, 2005) shed further insight on the dual role that dectin-1 may possibly play in polarizing the immune response. Dectin-1 is also postulated to play a role as a co-stimulatory molecule during the interaction between APCs and T cells by being able to bind to $CD4^+$ and $CD8^+$ T cells.

1.2.4.3.3 Dectin-2 (C-type lectin domain family 6, member A – CLEC6A)

Dectin-2 is a recently discovered C-type lectin about which not much is known. Dectin 2 expression is characteristic of the arrival of monocytes being recruited into inflammatory sites (Taylor *et al.*, 2005). This expression related to these activated macrophage/monocytes is unrelated to the stimulus initiating the inflammation. Similar to dectin-1, dectin-2 shows a tendency to form a spliced variant transcript that has 34 amino acids deleted (and consequently has a truncated extra cellular stalk as in Figure 1.6). This stalk is reported to be necessary for dimerization, thus the variant is supposedly incapable of dimerization. This may have a functional bearing on the ability of the truncated variant to participate in immune mechanism involving other molecules and/or PRRs. Dectin-2 has also been implicated in the ultraviolet (uv) induced tolerance (Aragane *et al.*, 2003) possibly via regulatory T cells or by modulating Langerhans cell function in a uv dose-dependent down-regulation of dectin-2 (Gavino *et al.*, 2005; Sakaguchi, 2004).

1.3 PRR polymorphisms and immune disorders

In view of the fact that the amino acid composition of a ligand binding site is critical to its functionality and specificity; mutations within these sites could potentially affect their response to pathogens and a host's predisposition to infection and/or disease. Similarly, mutations in the signal transduction sites would affect the ability of successful ligand binding achieving optimal downstream immune effects. Genetic predisposition is usually multigenic in nature and will involve several similar function-linked genes. Together mutations in these genes would be responsible or account for the risk to develop a particular disease.

Recent studies have shown numerous polymorphisms associated with the PRRs. A number of these mutations have been shown to affect the response of the PRR to the usual ligands and some have been associated with specific disease states. Increased susceptibility to Legionnaires disease was shown by Hawn and co-workers (Hawn *et al.*, 2003) ascribed to a TLR5 stop codon polymorphism that led to a defective innate recognition of the causative pathogen. Not all polymorphisms are detrimental to immune mechanisms; Hawn and co-workers (Hawn *et al.*, 2005a) demonstrated a protective association with human TLR 4 SNP A896G ($D^{299}G$) to Legionnaires disease caused by the Gram negative bacterium *Legionella pneumophila*. Resistance to systemic lupus erythematosus (SLE) via a TLR5 stop codon polymorphism was also demonstrated by the same authors (Hawn *et al.*, 2005b). A TLR4 mutation $Asp^{299}Gly$ has been described associated with poor LPS sensing leading to inflammatory bowel diseases, Crohn's disease and ulcerative colitis (Franchimont *et al.*, 2004). Imahara and co-workers (Imahara *et al.*, 2005) however, did not find any hyporesponsiveness to LPS and concluded that this mutation is not a determinant in endotoxin sensitivity. The same $Asp^{299}Gly$ mutation has been associated with decreased risk of atherosclerosis (Kiechl *et al.*, 2002), giving credence to the infectious basis of autoimmune disease. TLR2 recognizes the largest variety of described PAMPs amongst the TLRs and numerous polymorphisms have been described; TLR2 $Arg^{677}Trp$, has been associated with lepromatous leprosy (Bochud *et al.*, 2003; Kang and Chae, 2001), TLR2 $Arg^{753}Gln$ with increased susceptibility to

tuberculosis (Ogus *et al.*, 2004). The TLR2 $Arg^{753}Gln$ mutation is also associated with decreased immune responses to peptides from other bacteria and may predispose people to staphylococcal infections (Lorenz *et al.*, 2000). However, the lack of TLR2 in mice has been shown to make them more resistant to fatal *Yersinia* infections. This further goes to show that mutations in an innate immune receptor can mediate either beneficial or deleterious inflammatory responses that will vary with different ligands or pathogens.

Other mutations in different parts of the TLR signalling pathways have been described. However, in view of the importance of TLRs, a complete deficiency in one of the main TLR pathways is probably not compatible with life (Netea *et al.*, 2004).

A frame shift mutation at nucleotide 3020 of the human CARD15 gene, the 3020insC, has been described (Ogura *et al.*, 2001) in the gene's LRR region in association with Crohn's disease (Hugot *et al.*, 2001). This mutation in the gene has been ascribed to impaired nuclear factor-kappa B (NF-κB) response in mutant type immune hosts. The 3020insC results in the change of amino acid that leads to a premature stop codon. The resultant truncated CARD15 has 1007 amino acids instead of 1040 amino acids (see Figure 1.5) of the normal CARD15 protein (Ogura *et al.*, 2001). This mutation that results in the loss of the last 33 amino acids of the C-terminal leucine-rich region of the protein is postulated to affect the ligand binding ability of the molecule leading to possible inability to recognize its natural ligand. This then prevents downstream immune signalling. Mutations elsewhere on the CARD15 (not in the LRR region) are thus postulated to have other effects not related to immune recognition (Miceli-Richard *et al.*, 2001).

Great controversies rage on whether CARD15 contributes to the pathogenesis of Crohn's disease in humans. The basic question is; do the CARD15 mutations lead to a 'loss-in-function' or 'gain-in-function' phenotype? (Kelsall, 2005). Using genetically modified mice, conflicting results have been published by two groups on the role of mutant CARD15 proteins in regards to the predisposition to Crohn's disease. Kobayashi and co-workers (Kobayashi *et al.*, 2005) concluded that the

mutations led to loss of function whilst Maeda and colleagues (Maeda *et al.*, 2005) concluded that the mutations lead to a 'gain of function' phenotype. A third study by Watanabe and colleagues (Watanabe *et al.*, 2004) also using CARD15 knock out mice concluded that CARD15 has a regulatory function rather than a bacterial sensing function (mechanism summarised in Figure 1.7). Using more specific disruptions of the CARD15 gene Pauleau and Murray (Pauleau and Murray, 2003) concluded that CARD15$^{-/-}$ macrophages were not responsive to MDP, but that CARD15 does not have any specific regulator function in macrophage activation. However, work done by Berrebi and co-workers (Berrebi *et al.*, 2003) found an increased expression of CARD15 in epithelial cells of patients with inflammatory bowel disease (IBD). This would link inflammatory bowel diseases with cytosolic bacterial sensing.

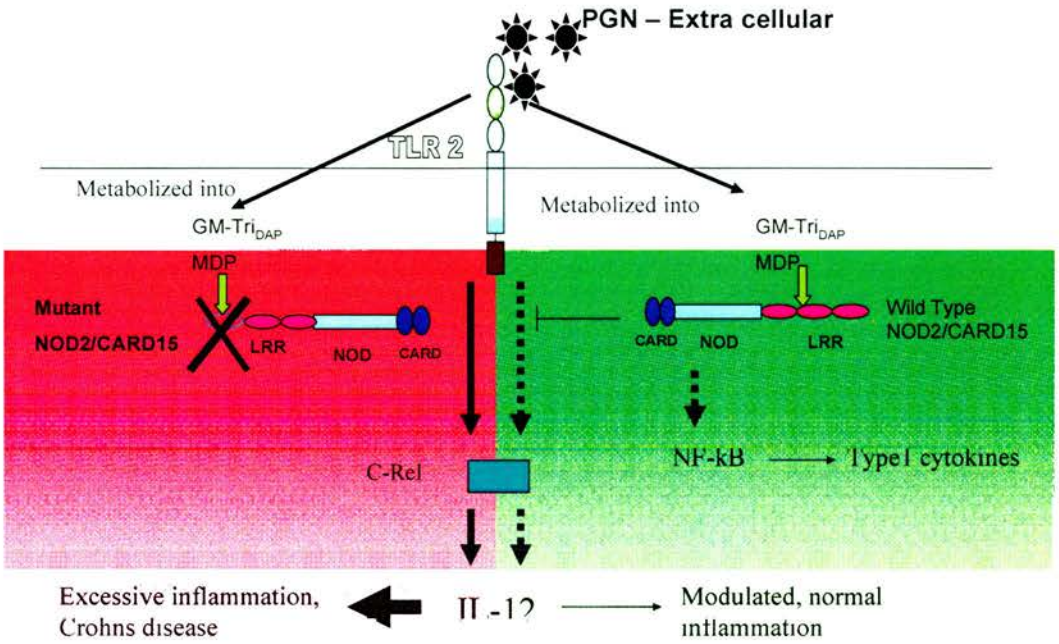


Figure 1.7 CARD15 mutation and excessive Il-12 production.

Schematic representation of proposed mechanisms involved in CARD15 mutation mediated hyper-inflammatory states in disease. On the right (green shaded) normal CARD15 modulates the TLR2 signalling leading to controlled inflammatory response to TLR ligation by PGN. On the left (red shaded) the mutant CARD15 (3020 InsC – see Figure 1.5) has a truncated LRR domain and is unable to sense MDP. It thus, does not have the modulatory effect on the TLR2 signalling and this leads to excessive IL-12 production and a hyper-inflammatory state.

1.4 Cells of the Immune System

The immune system, tasked with the responsibility of protecting a host against pathogenic challenge, has an array of cells found in the blood, lymph and tissues. In humans, these cells have a common progenitor, the pluripotent haematopoietic stem cell in the bone marrow or foetal liver. All cells of the immune system are produced from here continuously and the production is antigen-independent. This pluripotent progenitor gives rise to the common lymphoid progenitor (that gives rise to T cells, B cells and plasmacytoid dendritic cells) and the common myeloid-erythroid progenitor (that gives rise to eosinophils, neutrophils, basophils, monocytes, myeloid dendritic cells).

Figure 1.8 shows a schematic representation of the development of human white blood cells from a common haematopoeitic progenitor. However, recent evidence is disputing the distinct 'committed two lineage model' (Kawamoto *et al.*, 1997). The same principal author (Kawamoto, 2006) proposes a closer relationship between the lymphoid and myeloid lineages whereby common lymphoid progenitors retain some myeloid potential and are thus termed common myelo-lymphoid progenitors. PRR expression profiles based on lineage have been described with the myeloid derived cells having the widest range (Taylor *et al.*, 2005).

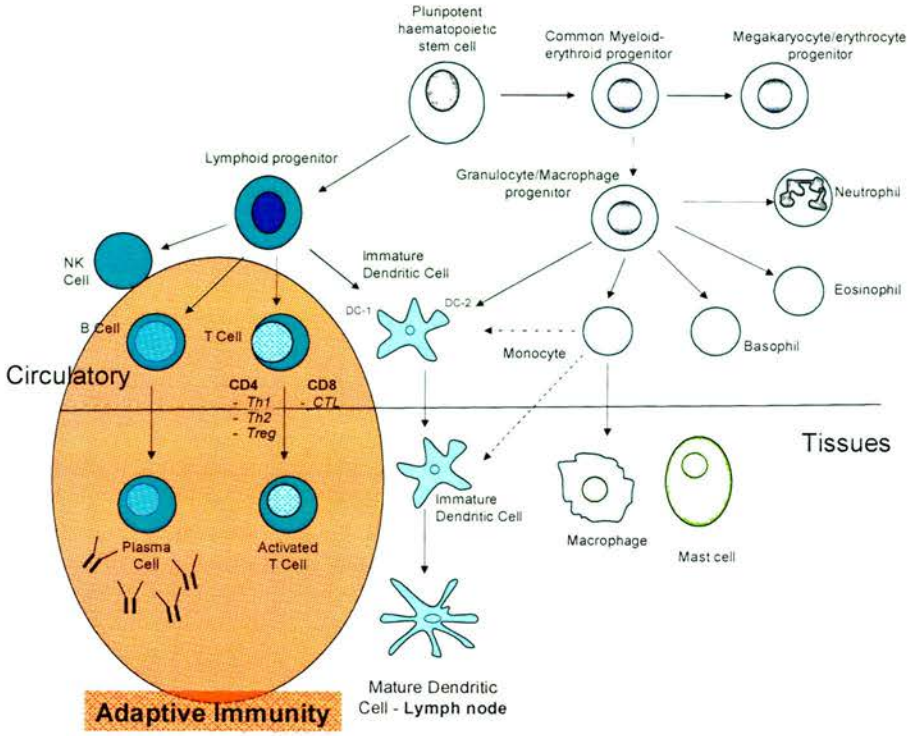


Figure 1.8 Development of mammalian white blood cells

Schematic overview of the development of leucocytes from their stem cell derivative. The bone marrow derived pluripotent haematopoietic stem cell divides into the lymphoid and common myeloid-erythroid progenitor that give rise to the lymphoid and myeloid lineages respectively. Dendritic cells are derived from both lymphoid and myeloid progenitors.

1.5 Dendritic Cells

Dendritic cells (DCs) are bone marrow-derived cells that possess both lymphoid and myeloid stem cell derivation - see Figure 1.8. DCs are significantly more potent than any other APCs in their ability to activate immunologically naïve T cells to differentiate into effector cells (Banchereau and Steinman, 1998; Hart, 1997; Lanzavecchia and Sallusto, 2001; Stephens *et al.*, 2003). DCs are highly migratory cells, and although making up less than 1% of the total mononuclear cells in an animal, they are present in their immature form in all tissues including the CNS. DCs act as peripheral sentinels (possessing the unique capacity to sample all sites of potential pathogen entry), detecting, deciphering and responding to signs of antigen invasion, processing antigen and then moving into lymphoid organs where they activate T cells. DCs are necessary for the initiation and amplification of the immune responses due to their potent and unique ability to take up and process antigen (Ag) from the peripheral sites to the lymph nodes. They usually take up antigen by micropinocytosis, receptor-mediated endocytosis and phagocytosis, but Ag may also be acquired by direct infection of the DC with a pathogen or binding the immunoglobulin Fc or complement receptor. Numerous stimuli may induce the activation, maturation, and migration of DCs. These stimuli include whole pathogens, PAMPs on pathogens, and cytokines such as IL-1 and TNF-α produced by other immune cells. As DCs migrate towards the lymph nodes, they mature; lose their capacity for endocytosis and become more immunogenic (possessing more dendrites and expressing a higher number of co-stimulatory & MHC peptides molecules). By virtue of these properties, DCs are able to regulate not only the quantitative nature, but also the qualitative nature of an immune response, mediate Th1/Th2 polarization and immune tolerance.

DCs are a heterogeneous cell population that make up a key cellular element of the innate immune system and also have the function of bridging the innate and adaptive immune system (Kelsall *et al.*, 2002; Moll, 2003; Reis e Sousa, 2004). In the lymph nodes DCs present the processed antigen to naïve T lymphocytes in order to prime and stimulate them. DCs are responsible for directing the naïve Th cells to

differentiate into Th1, Th2 or T regulator effector cells (Kapsenberg, 2005; Mazzoni and Segal, 2004; Moser and Murphy, 2000) and thus have the capacity to polarize the immune response accordingly or lead to immune tolerance (Jonuleit *et al.*, 2001). The cytokine milieu secreted by DCs is able to exert polarizing effects on Th cell polarization by exerting selective influence on the development of Th0 cells. DCs are also paradoxically responsible for the suppression of the immune system via Ag specific T suppressor cells becoming tolerogenic and inducing T helper cell anergy. Other than the modulation of T cell function, DCs are also known to recruit other inflammatory cells and promote angiogenesis during the process of inflammation. Thus, in addition to the teleological task of DCs priming naïve T cells, they have recently been shown to affect the function of natural killer cells during infection (Andrews *et al.*, 2005). *In vitro* studies have demonstrated that mature DCs are able to modulate NK cytotoxicity and IFN-γ production. In turn, activated NK cells seem to enhance DC maturation and IL-12 production. Thus DCs have a unique function in both priming an adaptive immune response and also in the induction of tolerance. DCs have however, also been implicated in the trafficking and spread of various intracellular pathogens such as mycobacteria (Humphreys *et al.*, 2006).

Several DC subsets with different biological characteristics have been identified in humans and mice. These subsets include the conventional myeloid DC and the lymphoid DC (Hart, 1997; Shortman and Liu, 2002) based on their developmental derivation (see Figure 1.8). DCs may also be classified according to their location in different tissues. They are called Langerhans cells (LC) in the epidermis, dermal DCs in the dermis and thymic DCs in lymphoid organs. Murine DCs can further be classified according to CD4 and CD8α expression. In the mouse spleen, 5 distinct sub-sets may be classified based on these classification criteria. DCs may also be classified according to their maturation status. DCs may be classified as mature or immature based on expression of co-stimulatory molecules b7 (CD80 and CD86), CD40 and MHC class II. Immature DCs are found predominantly in the periphery but some may migrate and be found in secondary lymphoid tissues (Shortman and Liu, 2002). This large array of classification criteria for DCs does not translate across species and the in depth description of these numerous sub-populations in multiple

species is beyond the scope of this thesis. Ovine DC subsets are less well defined as compared to human and murine subsets and the best-characterized ruminant DC populations are the SIRPα (CD172a) positive and negative populations (Brooke *et al.*, 1998; Howard *et al.*, 1997). These subsets have different cytokine profiles (Stephens *et al.*, 2003), and capacities to induce T cell responses (Hope *et al.*, 2001) with the $CD172a^{+}$ DCs preferentially producing IL-10 and potently stimulating of both CD4 and CD8 T cell proliferative responses, while the $CD172a^{-}$ DC population preferentially produce IL-12 with poor CD8 Tcells stimulation.

Different DC subsets have the capacity to interact with and influence other cells such as B and T cells, and NK cells of the immune system to modulate immune responses. Dendritic cell subsets differ in their ability to prime Th1 Th2 responses (Pulendran *et al.*, 1999; Voisine *et al.*, 2002; Yrlid and Macpherson, 2003). In humans myeloid DCs (DC1) are proposed to trigger Th1 type immune responses whilst activation of plasmacytoid DCs (DC2) is proposed to culminate in Th2 type responses. In the mouse CD8+ DCs promote Th1 type immune responses via the synthesis of IL-12 while other subsets are not able to make IL-12 and consequently promote Th2 type immune responses (Pulendran *et al.*, 1999).

Development of the DCs from their haematopoetic progenitors is poorly understood but DC populations are of well-known heterogenicity (Jung Hoon Ahn *et al*, 2002). In addition, the underlying genetic programs that determine lineage commitment and differentiation of DCs are largely unknown and likely involve the selective activation and/or repression of specific genes (Hacker *et al.*, 2003; Richards *et al.*, 2002). Immature DCs also express a variety of PRRs including members of the TLR family (Akira and Hemmi, 2003; Heil *et al.*, 2004; Iwasaki and Medzhitov, 2004; Moser, 2004; Reis e Sousa, 2004; Werling *et al.*, 2006) and cytokine profiles (Stephens *et al.*, 2003) and are therefore conferred with a diverse capability to interact with pathogens and these receptors allow DCs to recognize PAMPs and thus detect signs of infection by decoding the patterns of self and non-self. This leads to efficient activation and maturation of DCs and induces their migration into the T cell zone of lymphoid organs (Moser, 2004). The identification of PRRs as being a critical factor in controlling DC activation and maturation has led to the premise that these

receptors may regulate Th1/Th2 polarization (Sun *et al.*, 2005). Subset-specific PRR expression profiles of DCs have been implicated as a mechanism for differentially sensing antigens and are thought to determine Th1/Th2 polarization. Although specific immunological functions as well as unique repertoires of PRRs have been attributed to these subsets, the distinctions are seldom absolute and each appears to be capable of responding to a variety of microbial stimuli (Manickasingham *et al.*, 2003; Sher *et al.*, 2003). Several studies however, challenge this view as being rather simplistic. Contrary to the originally held notion, it has been postulated that the ability of DCs to polarize specific T helper cell response *in vivo* does not seem to be an inherent function of specific DC subsets, but that of a range of possible responses dependent on the nature of stimuli received by DCs, antigen dose, activation state of the DC, and the milieu in which DC interaction occurs (Anderson *et al.*, 2004; Kaisho and Akira, 2004; Manickasingham *et al.*, 2003).

Dendritic cells have been described as the most useful cell tool for elucidating TLR function (Kaisho and Akira, 2004), taking into account their pivotal role in linking the innate and adaptive immune system (Hoebe *et al.*, 2004). Few direct functional comparisons of the PRR expression in different subsets of DCs in veterinary species exist (Werling *et al.*, 2006) and there are none for the sheep. Thus, the elucidation of the PRR expression in the ovine immune system cells and tissues would be very useful for the understanding of the potential role that PRRs and innate immunity contribute to host-pathogen interactions outcomes.

Ruminant and pig lymphatic canulation has provided a unique and powerful tool that allows the analysis of DCs *ex vivo*. This method available in these species, allows for the collection of pure populations, in sufficient quantities, of DCs in their almost natural state for functional immunological studies to answer fundamental questions about *in vivo* DC biology (Haig *et al.*, 1999). DCs show extreme phenotypic and functional plasticity and any experimental manipulation such as culture is likely to greatly affect direct relevance of such findings to *in vivo* DC biology. This tool thus, allows access to 'steady state' DCs as they exist *in vivo* which in not possible in humans and laboratory mice.

1.6 Peripheral Blood Mononuclear Cells (PBMCs)

1.6.1 T Lymphocytes (T cells)

T lymphocytes are bone-marrow derived cells that mature in the thymus. They constitute the cellular wing of the adaptive immune system and are responsible for recognizing antigen peptides that are bound to MHC proteins and presented by APCs. T cells that haven't encountered antigen are referred to as naïve T cells ($CD45RA^+$). Based on differences in MHC class restriction, effector function and type of accessory molecule use, two main types of T cells have been traditionally described; helper T cells and cytotoxic T cells. Through their antigen-specific cytotoxic action, T cells also play a vital role as effector cells. Suppressor T cells are another subset of T cells whose function is mainly in the maintenance of homeostasis and tolerance and include regulatory T cells (Tregs) and Th3 cells. A recently described T cell sub type is the Th17 type that is responsible for protective effector and homeostatic immune responses (Harrington *et al.*, 2005; Harrington *et al.*, 2006; Park *et al.*, 2005). Following activation, clonal expansion and death, some memory T cells ($CD45RO^+$) are retained that are able to respond more proficiently to the same antigen. T cells are known to express PRRs, however, the functional expression of PRRs on T cells is not well characterized. The expression of PRRs on T cells would imply that these cells have a direct innate immune function and would recognize naïve PAMPs independent of APCs antigen processing.

1.6.1.1 CD4 T cells (T helper cells)

T helper (Th) cells recognize antigen in the context of MHCII presentation. The main function of these cells is cytokine secretion, macrophage activation and provision of helper signals for B cells and CD8 T cells. T helper cells may be classified based on their cytokine production. Th1 cells secrete copious amounts of IFNγ and associated pro-inflammatory cytokines such as IL-12 whereas, Th2 cells secrete anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 which tend to promote IgA and IgE antibody production by B cells.

T helper cells are thus a vital regulator of immune response via polarization of a stimulated naïve T cell towards Th1, Th2 or regulatory phenotype. $CD4^+$ cell polarization is thus important in determining the outcome of disease as imbalances in Th1/Th2 type responses are known to be crucial in the pathogenesis of certain allergic and autoimmune diseases (Liblau *et al.*, 1995; Neurath *et al.*, 2002; Romagnani, 2004).

1.6.1.2 CD8 T cells (Cytotoxic T cells)

Cytotoxic T cells recognize antigen in the context of MHC class I presentation on infected cells or on APCs. The main function of these cells is the direct killing of cells infected with intracellular organisms such as viruses, protozoan pathogens and also killing of neoplastic cells. CD8+ T cells are able to achieve these anti-microbial functions by the release of cytolytic molecules via perforin and Fas pathways that kill infected cells and by activating and recruiting other immune cells.

1.6.2 B Cells

B cells are lymphoid-derived cells that develop in the bone marrow and whose principal task is the production of protective high-affinity immunoglobulins against invading pathogens. Following activation, B cells divide into plasma cells and memory B cells. Their major function is thus participating in adaptive immune responses; however, B cells also have an innate immune function by secreting IgM following innate immune receptor ligation (Milner *et al.*, 2005). B cells have been shown to express an array of receptors (Bishop and Hostager, 2001) including PRRs (Dasari *et al.*, 2005; Hornung *et al.*, 2002) and are able to recognize naïve antigen and thus act as APCs (Constant, 1999; Rodriguez-Pinto, 2005).

1.6.3 Monocytes/Macrophages

Monocytes circulate in blood and they move from the circulation into tissues where they may be generally called tissue macrophages. They are called more specialized names in different tissues such as microglia (brain), mesangial cells (kidney), osteoclasts (bone) and Kupffer cells (liver). These cells express the myeloid receptor CD14, immunoglobulin and complement receptors and have the ability to take up particulate antigen and dead/apoptotic cells by phagocytosis. These cells are consequently also able to act as APCs for T cell activation. Monocytes are also able to differentiate into fully functional DCs (Randolph *et al.*, 1998; Randolph *et al.*, 1999; Randolph *et al.*, 2002). Besides taking part in phagocytosis and antigen presentation, macrophages also secrete cytokines such as IL-1, IL-6, IL-10, IL-12 and TNFα during an inflammatory process and thus able to modulate immune responses.

Other cells such as gamma delta T cells (γδ T cells) and NK cells take part in innate immune mechanisms. Gamma delta T cells have been shown to respond directly to PAMPs (Hedges *et al.*, 2005) and are thus postulated to be able to drive an immune response independently of APCs. NK cells are large cytotoxic lymphocytes that, unlike B cells or T cells, do not secrete antibodies nor express T cell receptors at their cell surface. NK cells are important innate immune cells and represent a first line of defence to infections with viruses or intracellular organisms, and 'alterations of self' and are unique in that they are cytotoxic in the absence of prior stimulation.

1.7 The Epitheliae (Skin and Mucosae) - The first line of defence

The skin and mucosae have pleiotropic responsibilities that include forming the first line of physical defence, sensation, selective permeability and secretory/absorption functions. Other than the physical/mechanical barrier that these surfaces provide, complex immunological processes take place that provide a link between the adaptive and innate immune systems. These surfaces are sites for antigen uptake, processing, presentation, co-stimulatory signalling and tolerance induction. An adult human has approximately 400 m^2 of mucosal surface (predominantly that of the gastrointestinal, respiratory and urogenital tracts) and this presents a very large area for potential pathogen entry. Different epithelial surfaces each have a unique population of 'normal' resident microflora, the commensal organisms, which have evolved to co-exist with the host in a beneficial and symbiotic manner.

Commensal organisms and the mucosal immune system co-exist in a delicate balance. This is achieved by the multifaceted role that the immune system plays in protecting against auto-pathology whilst maintaining local immune proficiency.

1.7.1 Immunity at the Skin

The skin is made up of keratinized stratified squamous cell epithelium. This unique tissue is arranged in layers with the top most layer being made up of dead keratinized cells and most of the intervening cells are not in contact with the basement membrane. The skin represents a principal aspect of innate immune defense against disease, firstly as an anatomical/physical barrier to infection and secondly as a site for the induction of adaptive immune responses. The skin is the largest and most directly exposed boundary with the externum. However, despite its obvious daily exposure to the environment, widespread, persistent skin infections are rare. To maintain this healthy status poses unique demands on the local immune system of the skin to be able to withstand and respond rapidly and effectively to numerous challenges.

In order for the skin to function effectively, it recruits immune system resources from non-cutaneous sources such as lymphoid and myeloid cells from blood when such a need arises. The superficial epidermal layer of skin is underlined by a layer of epithelial cells known as keratinocytes. Keratinocytes are important innate immune cells in the skin and may function as 'non-professional' APCs and keratinocyte-derived cytokine mediated cellular communication leads to the recruitment of other cell types during pathogen challenge or cutaneous insult. Keratinocytes are known to express TLRs (Kollisch *et al.*, 2005) and ligation may lead to β-defensin release via TLR signalling (Sumikawa *et al.*, 2006). Other cells reside in the skin and include vascular derived leucocytes, dermal fibroblasts, Langerhans cells (LC), dermal dendritic cells, mast cells. Typically, blood monocytes may be recruited in a steady state or at an accelerated rate (during antigenic challenge) to replenish dermal DCs. A newly described $CD1a^{+}$ $CD207^{-ve}$ non-Langerhans dermal APC population in the human skin (Angel *et al.*, 2006) is also involved in cutaneous mediated immune response.

1.7.2 Immunity at Mucosal Surfaces

Mucosae make up the innermost layer of the organs that they line and come in direct contact with lumen contents. The mucosa is structurally divided into three layers; epithelium, lamina propria and *muscularis mucosae*. Unlike the skin that has a dead keratinous outer layer, all layers of the mucosal surfaces are made up of live cells and epithelial cells express PRRs and may initiate immune responses in response to pathogens. However, once mucosal epithelial integrity has been breached, more concereted inflammatory immune responses are initiated to prevent systemic infection.

The immune system at the mucosal surface is divided into two structurally and functionally distinct sites. The first are those that take part in antigen recognition, uptake and processing. These sites tend to be rich in lymphoid tissue, usually organized as mucosae associated lymphoreticular tissue (MALT), and APCs, T and B cells. Peyer's patches in the terminal ileum are an example of MALT and are

usually specifically called gut-associated lymphoreticular tissues (GALT). The second sites are those that take part in the effector functions of the immune system.

1.7.2.1 Immune mechanisms in the Gastrointestinal Tract (GIT)

The gastrointestinal tract possesses the largest reservoir of macrophages and lymphocytes in the body and also makes up the largest mucosal surface interfaced to the environment.

Gut contents are a heterogeneous population of micro organisms made up of symbiotic commensals and potentially pathogenic ones and also ingested food. The mucosal epithelia have the challenging task of deciphering the vast amount of antigentic information derived from gut contents and translating this into appropriate immune clearance or tolerance. The exact mechanisms by which the GIT immune system achieves this daunting task are yet still unknown. However, dysregulation in the immune balance leads to auto immune diseases such as inflammatory bowel disease (Crohn's disease, ulcerative colitis), and allergic gastroenteropathy. Peyer's patches make up the most specialized and organized gut-associated lymphoid tissue (GALT) where antigen recognition and processing take place.

At least four mechanisms are available by which immune sampling takes place and antigen may be delivered from the gut lumen to the sub-epithelial immune cells via three mechanisms (summarised schematically in Figure 1.9);

dendritic cells - specialized DCs sample the lumen contents by passing their dendrites between epithelial cells, whilst maintaining tight junction integrity. These specialized trans-epithelial dendrites carry out 'steady state' sampling and their activity increases in bacterial infections of the GIT. DCs bearing trans-epithelial dendrites are limited to the terminal ileum.

enterocytes - Intestinal epithelial cells may become infected and die by apoptosis. Sub-epithelial DCs will be able to take up such apoptotic cells and acquire antigen. Intestinal epithelial cells are also able to transport antigen from the lumen to the sub-epithelial space via a process known as transcytosis.

Direct access – Antigen may be delivered to sub-epithelial immune cells via disruptions in GIT surface mucus lining and the integrity of the epithelium. The tight junctions between intestinal epithelial cells (IEC) may be disrupted and allow leakage of intestinal contents into the sub-epithelial cells and access the immune system there.

Multi-fenestrated (or microfold) epithelial cells (M cells) – These are found on the epithelium of Peyer's patches, organised lymphoid follicles and scattered independently in the rest of the GIT epithelium. M cells engulf antigen by endocytosis and transport the antigen across the epithelium by transcytosis to be presented to APCs in the sub-epithelial space for downstream immune response. M cells are adapted for this function by lacking surface glycocalyx and not secreting mucus. Unlike intestinal epithelial cells, they are thus in direct contact/close proximity with lumen contents.

The main effector site for GIT immune mechanisms is thus, the lamina propria. It normally has a high number of DCs, macrophages, T cells, B cells, plasma cells, mast cells (see Figure 1.9). The non-ciliated simple columnar epithelium of the lower GIT is made up of a single layer of IECs with an underlying lamina propria. The lamina propria contains most of the immune cells. This anatomical arrangement has minimal resistance to injury and healing tends to be difficult when substantial portions of the epithelial lining have been eroded.

The GIT also discharges other innate immune function by virtue of a) the low pH in the stomach is anti-microbial b) secreted mucus (containing IgA) as well as other secreted components such as enzymes and bile have anti-bacterial effects and c) commensal microbes preclude the overgrowth of potentially pathogenic microbes in the GIT.

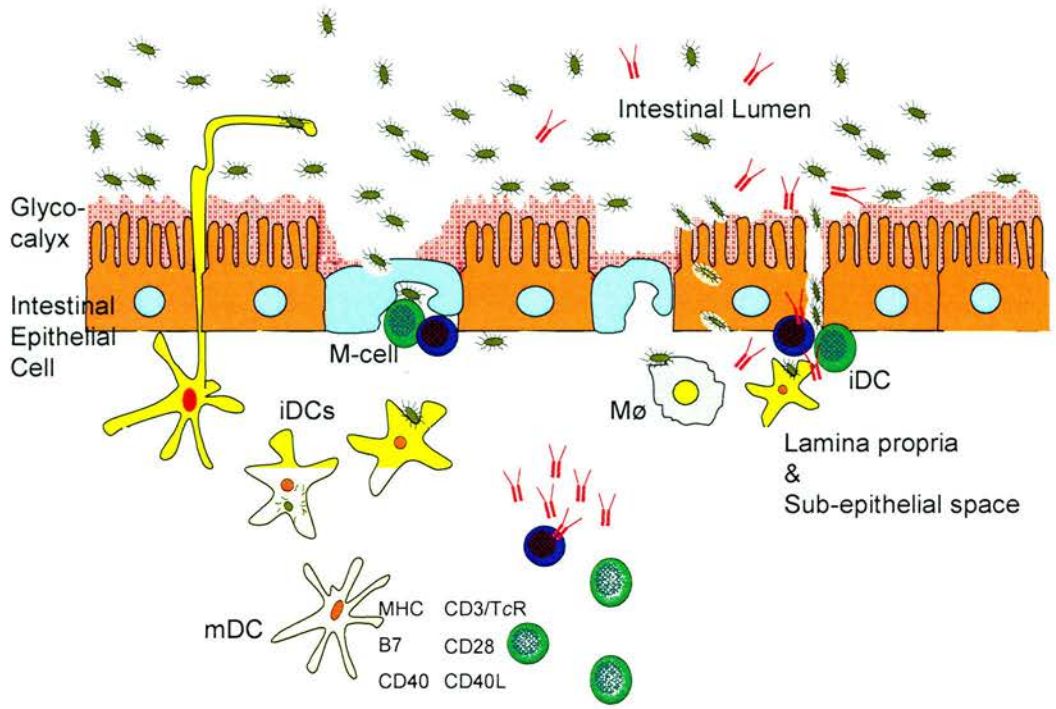


Figure 1.9 Mechanisms of Immune sampling and immune mechanisms in the gastrointestinal tract

Mechanisms of immune sampling in the GIT and components of the local immune system. The GIT lumen is separated from the lamina propria by a single layer of intestinal epithelial cells (IEC). The lamina propria contains B cells, T cells, APCs such as DCs, and macrophages. Antigen can be delivered to the immune system in the lamina propria by specialized DCs with long dendrites, via M cells, directly via IEC cell tight junction disruption and by transcytosis. Plasma cells release IgA in the subepithelial space and into the GIT lumen.

Abbreviations: DC dendritic cell; iDC immature DC; mDC mature DC; Mø macrophage

Key

Antibody (mainly IgA)

B cell

T cell

GIT lumen microbe

1.7.3 Oral Tolerance – immunity towards GIT commensals

The intestinal mucosa must strike a balance between rapidly recognizing pathogenic organisms and rapidly eliminating them via regulated immune responses and maintain hypo-responsiveness to normal gut flora/commensals (Cario, 2005; Rakoff-Nahoum *et al.*, 2004). The intestinal immune system is exposed to antigens derived from food as well as those from commensal. It is however, in a state of immunological balance with these 'normal' antigens. Unlike pathogens, commensal microbiota cannot be effectively eliminated from the GIT by the immune system, thus persistent immune responses to these organisms may cause chronic inflammatory disorders. A point worth noting is that these commensal organisms that are in a state of immunological balance in the GIT, would lead to severe or even fatal disease when introduced to mucosae of other organs such as the lung. Like pathogens, commensal organisms also possess PAMPs but do not normally elicit severe immune reactions. The reason for this is not understood but several theories have been advanced. Intestinal epithelial cells have been shown to express LPS related PRRs such as CD14, TLR4 and MD-2, in very low levels. This would therefore make them non-responsive (or hypo-responsive) to the LPS derived from gut micro flora. Responses triggered by LPS receptors tend to be inflammatory - but inflammatory reactions are contra-indicated at mucosal surfaces as they are tissue damaging and such damage tends to be difficult to resolve and results in erosive lesions. Macrophages in the gut also do not express significant amounts of certain PRRs such as CD14 (Abreu *et al.*, 2001; Smith *et al.*, 2001; Smythies *et al.*, 2005). Receptor complexes are also located away from potential sites of interaction with gut microflora. They may be located intracellularly instead of on the plasma membrane, a strategy thought to further protect against continuous inflammation or may be located on the basolateral aspect of epithelia whereby the epithelium has to be breached in order for them to have any contact with gut flora. The above strategies may be used for essential co-receptors, where the PRRs may have contact with the microflora, but the co-receptors such as CD14, MD-2 that are required for sensing are either absent, lowly expressed or present in a different physical location. The GIT mucosae are also known to have tolerogenic DCs (Weiner, 2001).

Gut microflora reside in the lumen of the gut and not directly on the epithelial cells. The physical separation of gut epithelial cells from lumen contents by the secreted mucus layer also limits epithelial reactivity. The mucus layer also contains anti-microbial peptides such as α and β-defensins. In contrast, pathogenic bacteria have developed virulence factors that confer them the ability to actively disrupt and penetrate the mucus lining to gain access and attach to the intestinal epithelial cells. This brings these organisms into direct contact with PRRs expressing IECs, macrophages, monocytes and dendritic cells and other immune system components. This results in appropriate immune responses aimed at eliminating or containing these microbes.

1.8 Ovine Paratuberculosis (Ovine Johne's Disease - OJD)

Paratuberculosis, an intestinal mycobacteriosis, also known as Johne's disease (JD), is a chronic, progressive granulomatous enteritis predominantly of ruminants caused by the bacteria *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) (basonym *M. paratuberculosis*). *M. a. paratuberculosis (Map)* causes granulomatous inflammation in the intestine (predominantly ileum) and associated draining mesenteric lymph nodes in ruminants. It usually gains entry into the body after oral ingestion and passage through the intestinal mucosa. Animals usually get infected as less-than-six-month-old neonates (kids, lambs, calves) but do not develop clinical signs until they become adults (average - cattle; 2-5 years, sheep and goats *ca* 2 years) and is usually ultimately fatal. The progression of the disease has four stages; silent infection, a protracted sub-clinical infection, clinical and advanced terminal clinical disease. During the protracted sub-clinical phase, the *Map* infection seems to be under control, but soon progresses to severe clinical disease with clinical signs related to a protein losing enteropathy.

Two discrete microscopical forms of pathology are described, related to a high or a low degree of mycobacterial colonization (termed as "multibacillary" or "paucibacillary"). Each of these forms has a characteristic type of cellular infiltrate in the terminal ileal mucosa and submucosa.

Paucibacillary (tuberculoid) - granuloma formation, high lymphoid cell, low macrophages, low bacterial counts

Multibacillary (lepromatous) - high bacterial counts, diffuse lesions, high macrophages

Sheep disease will have both pauci and multi, whilst cattle tend to be mostly lepromatous. Sheep will have chronic weight loss but rarely diarrhoea. *Map* strains of sheep and cattle disease have been shown to be different and can be distinguished by PCR (Collins *et al.*, 2002) but this has not been shown to be an influencing factor in the subtle differences in clinical manifestation between sheep and cattle.

JD is not a disease exclusively of ruminants, many other species including dogs, pigs, horses, chickens and primates may be affected. *Map* has a well-defined tissue tropism for the intestine (Sweeney *et al.*, 2006) and corresponding mesenteric lymph nodes, even if administered parenterally. Even when given subcutaneously or intravenously, it preferentially goes to the terminal ileum and colon where it causes chronic inflammation.

1.8.1 Pathogenesis of OJD

Map preferentially invades macrophages that are found in the ileal Peyers patches, where the macrophages play a conflicting function of being the primary site of *Map* replication and also an important cell type involved in the development of immunity to eliminate the *Map*. As a consequence, the pathogenesis of *Map* at the intracellular level (in macrophages) is very poorly understood. Further, most of the information in literature on *Map* is deduced from *M. tuberculosis* and other Mycobacterial species in human and murine infections/experiments. Similarities between the immuno-pathogenesis of leprosy and OJD exist and there are striking similarities between the granulomatous lesions of ruminant Johne's disease and those in human leprosy (Modlin *et al.*, 1988). Both diseases have clinical forms that are highly polarized and the Th1/Th2 balance has an important role in determining disease progression and outcome. Granulomata formation is associated with the paucibacillary (tuberculoid) form that is characterized by a Th1-type response. Granulomata are formed as organized immune barriers that limit infection and control disease progression. Chiu and co-workers (Chiu *et al.*, 2004) proposed that granulomata are likely sites where innate and adaptive immune responses merge, regulated by dendritic cells. On the other end of the immune spectrum is the lepromatous (multibacillary) form that is characterized by a Th2-type response. This Th2-type response has a weak cell-mediated immunity and is thus unable to limit the mycobacterial proliferation and tissue damage. In humans, tuberculoid granulomatous lesions have a better clinical outcome than lepromatous lesions. Thus, the control of mycobacterial infections in humans is directly correlated to the granuloma type. Mechanisms that mediate the decision to mount either a Th1-type or Th-2 type immune response, and the resulting

clinical form are not well understood. However, TLR signalling seems to skew naïve T cells towards Th1-type response polarization (Trinchieri, 2003a) and has also been proposed to actually censor Th2 cell development (Sun *et al.*, 2005). Paradoxically, TLR signalling has also been shown to drive Th2 responses in certain instances (Re and Strominger, 2001). This is achieved by DCs via subtle differences in PRR signalling and temporal factors leading to pathogen specific cytokine milieu that will determine the Th1/Th2 balance (Jankovic *et al.*, 2001; Langenkamp *et al.*, 2000).

During the progression of OJD, it is known that the initial control of the early sub-clinical *Map* infection, 'reactive paratuberculosis' is due to the limitation of the infection by Th1 type (tuberculoid) granuloma formation. The effective immune response is characterized by a strong cytotoxic and proinflammatory action, but this gives way to a Th2-type response characterized by humoral immunity. The dynamics of the change from Th1 to Th2 immune response and overt clinical disease are poorly understood. It is however, known that the decision to differentiate into Th1 and Th2 CD4+ T cells is made early following infection and antigen recognition and processing for presentation. Koets and co-workers (Koets *et al.*, 2002) working with *Map*, hypothesized that the reduction in the number of protective CD4+ T cells leads to a lack of control of mycobacterial replication. This consequently, leads to the fulminating granulomatous enteritis that is typical of ruminant paratuberculosis. Zhang and co-workers (Zhang *et al.*, 1995) proposed that the progression in the mycobacterial infection is due to a diminished Th1 response *per se* and not an increase in the Th2 response. However, numerous authors report and increase in the number of infiltrating CD4+ cells (Bassey and Collins, 1997; Weiss *et al.*, 2006) in paratuberculosis lesions

The exact mechanisms involved in the shifting immune response to *Map* are unknown but are most likely multifaceted, involving antigen processing, antigen presentation, and tolerance induction.

Table 1.4 Morphology of human leprosy granulomas

	Tuberculoid	Lepromatous
Delineation	Well delineated	Poorly delineated
Intracellular Bacteria	Low bacterial burden	High bacterial burden
Macrophages	Epitheliod/multinucleate	Polygonal and histiocytic
Mineralization/Necrosis	Present	Absent

Adapted from (Hostetter *et al.*, 2005)

1.8.2 *Mycobacterium* and pattern recognition receptors

To date there has been no comprehensive and mechanistic dissection and understanding of how Mycobacterial infections proceed and how host defenses are marshalled against *Mycobacterium*. The comprehensive understanding of both successful and unsuccessful host defense mechanisms is a very important goal but it remains elusive. Quesniaux and co-workers (Quesniaux *et al.*, 2004) postulated that mycobacterial factors and released products such as lipoarabinomannan (LaM) contribute towards continued macrophage and DC stimulation via TLRs and other PRRs in cases of mycobacterial infections. Disparate findings exist on the role of PRRs in mycobacterial disease pathogenesis. For example, Sugawara and co-workers, (Sugawara *et al.*, 2003) using TLR2 and TLR6 knock-out mice, concluded that these TLRs are necessary for the recognition of mycobacteria but do not determine the outcome of pathology. It would also concur with Nicolle and co-workers (Nicolle *et al.*, 2004) who, working with knock-out mice concluded that TLR2, TLR4 and TLR6 are not necessary for long-term control of BCG mycobacteria. This is in contrast to other workers who concluded that TLR2 expression correlates with a better out come of disease in leprosy (Krutzik *et al.*, 2003).

1.8.3 Immunity development to *Mycobacterium* species and Immune evasion strategies by mycobacteria

The induction of a strong and appropriate innate immune response to invading mycobacteria is essential for the control of infection. *Mycobacterium tuberculosis* has however, been shown to survive the innate immune responses by suppression of inflammation. It has been postulated to use the tolerogenic effects of PRR signalling to facilitate its long term persistence (Pai *et al.*, 2003). *M. tuberculosis* uses numerous other mechanisms to evade host immune responses including the down regulation of MHC classII (Noss *et al.*, 2001) leading to poor antigen processing and manipulating phagosome maturation, reviewed by Flynn and Chan (Flynn and Chan, 2003). *M. tuberculosis* inhibits phagosome maturation into phago-lysosome and also prevents the acidification of the phagosome, thus maintaining a milieu that ensures their continuing survival in the engulfing macrophage or APC (Harding *et al.*, 2003). Mycobacteria may use PRR signalling to evade the immune system. The C-type lectin DC-SIGN has been postulated to be used by many viral organisms and *Mycobacterium* to evade immune recognition (van Kooyk and Geijtenbeek, 2003).

In mice, immunity to *M. avium* has been shown to be regulated by a host of other unknown MyD88 dependent factors in addition to the well-known TLR2 and TLR4 (Feng *et al.*, 2003). *M. tuberculosis* also causes immuno suppression in host's cells by secreting large quantities of glycosylated antigen.

Manca and colleagues (Manca *et al.*, 2001) show that different strains of *M. tuberculosis* strains have different capacities to induce Th1 responses and this has an impact on disease outcome. Strains of *Map* derived from different spps. may have different pathogenic effect on macrophages under in vitro conditions (Janagama *et al.*, 2006). This may have a bearing on the manifestation of clinical disease in sheep although no work has conclusively shown this.

The strong association between the incidence of Crohn's disease in humans and the culture of *Map* in these individuals (Grant, 2005; Naser *et al.*, 2004; Sartor, 2005), has led to speculation that *Map* may be zoonotic and pathogenic in humans under

certain conditions. This is a developing and still very controversial association with some authors finding no correlation (Ellingson *et al.*, 2003). Modern wildlife management practices of farming wild animals for profit or preservation, have now led to the increase of diseases that are by and large scarce in wild herds, including such diseases such as Johne's disease (Motiwala *et al.*, 2004). This changing epidemiological perspective of Johne's disease will inevitably bring new challenges to the control and attempts towards eradication of this economically and immunologically challenging disease.

1.9 Aims and Objectives

1.9.1 Background and Hypotheses

In order to understand the role that PRRs have in the immune system in sheep, the expression pattern of these PRRs in normal tissues and cells must be well defined. This would provide a baseline, from which deviations would indicate a shift into a diseased state or immune activation. The critical task was therefore to first identify PRR homologues in sheep as have been described in humans and other species and characterize their expression in different tissues, cells and disease situations. I hypothesized that all PRRs described in humans would all be present in sheep and also have similar functions and expression profiles in 'steady state' tissues and cells and in similar diseases as in humans and the mouse.

It is well documented that mammalian neonates are more susceptible to infectious challenge than adults and this is because they have an underdeveloped immune system. Most of the work on the underdevelopment of the immune system has been focused on the adaptive immune system development from birth (term or premature) compared with adults. It is however known that foetuses are able to respond to vaccinations. With the knowledge that the innate immune system drives adaptive immune responses, I undertook to investigate if this underdevelopment may be primarily due to an underdeveloped innate immune system and particularly due to a naïve pattern recognition receptor system. In order to unravel the potential to recognize antigens by the developing foetus, I undertook to comparatively quantify the PRRs mRNA expression in the skin and spleen of pre-term ovine foetuses and adult sheep. The premise being that since neonates are known to have under-developed immune systems, the PRRs mRNA expression in developing foetuses could be either very low or absent at birth and increases with age.

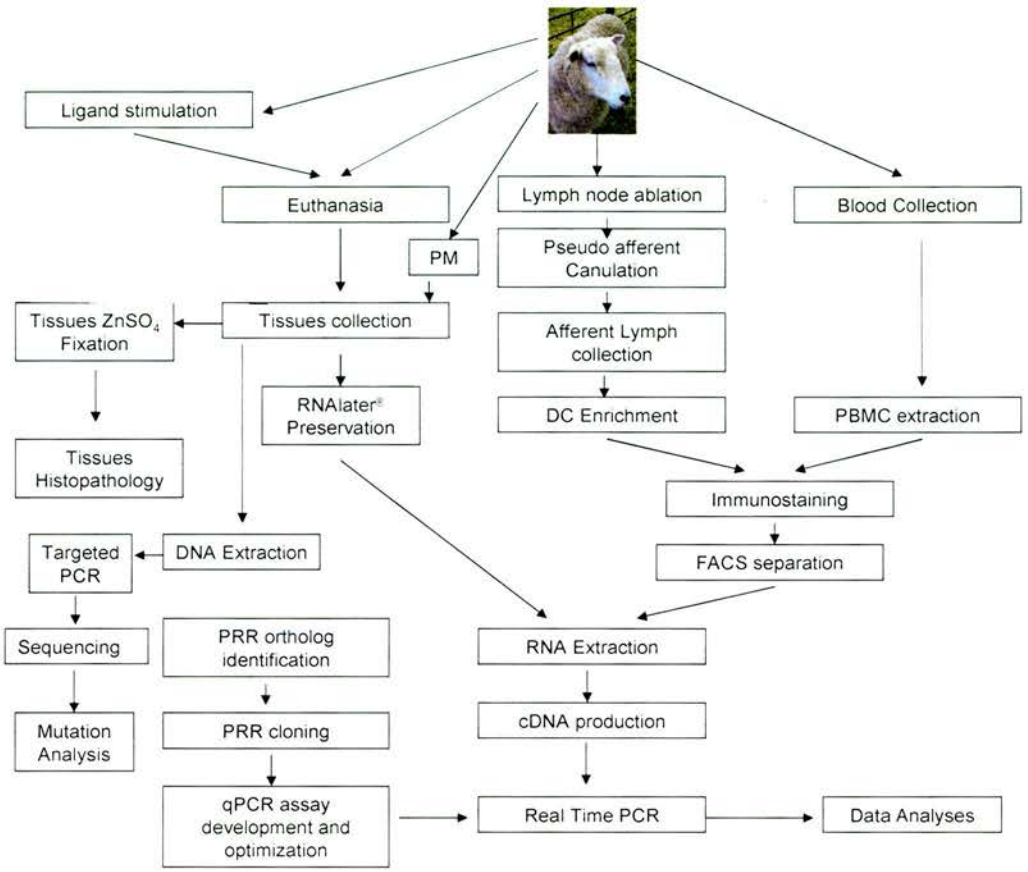
PRRs have been shown to be altered in disease states. PRRs are recognized to be pivotal in innate immunity development and innate immune mechanisms are known to be important for the control of *Mycobacterium* infections. I hypothesized that PRRs may be critical in determining outcome of *Map* infections and the resultant clinical forms of JD.

1.9.2 Specific Aims and Objectives of the Study

- To identify sheep homologues of selected PRRs described in humans and mice
- To elucidate the *in vivo* gene expression patterns of PRRs in 'steady state' ovine dendritic cell subset populations
- To characterize the existence and distribution of ovine PRRs in selected different cell types and tissue types in clinically healthy subjects.
- To characterize the expression of PRRs in ovine paratuberculosis. The hypothesis being that altered PRR expression during antigen recognition and processing (particularly TLR2 with its hetero dimers and associated accessory molecules) may account for the development of any of the three clinical manifestations of the disease in sheep.
- To characterize the expression of PRRs in LaM (a *M. paratuberculosis* associated ligand) stimulated skin in sheep.
- To characterize the expression of PRRs in the preterm foetal spleen and skin.

2 Chapter Two - Materials and Methods

Figure 2.1 Schematic overview of the experimental procedures carried out during the PhD study



Schematic overview of the experimental procedures carried out in this study

Abbreviations: DC, dendritic cells; FACS, flourescense activated cell sorting; PM, post-mortem; qPCR quantitative real time PCR;

2.1 Chemical and Reagents

All reagents used were molecular biology grade quality and obtained from reputable suppliers listed in Appendix I. The name of the manufacturer of each reagent is noted against the particular reagent. All oligonucleotide primers were synthesized by Sigma-Genosys, UK. The recipes for all commonly used buffers and solutions are in the appendix.

2.2 Sample collection and processing

2.2.1 Ethical approval - study animals

All animal experiments were approved by the University of Edinburgh's Ethical Review Committee and conformed to the standards and practices of the Animals (Scientific Procedures) Act 1986. All the experiments were carried out under Home Office project licence and personal licence PPL/60-3072 and 60/9994 respectively.

2.2.2 Normal ovine tissues

Samples were collected from clinically healthy adult ewes kept predominantly indoors. The ewes had previously been treated with the anthelmintic moxidexin (Cydectin®) eight weeks prior to euthanasia. All animals were euthanized humanely using intravenous pentobarbitone sodium and exsanguination. The anaesthetic was administered to effect terminal anaesthesia and once unconsciousness was achieved, the animals were exsanguinated via carotid artery bleeding. Tissues were collected immediately afterwards.

Approximately one cm^3 of each of the tissues; mesenteric lymph node, pre-scapular lymph node, skin, kidney, lung, spleen and urinary bladder was collected and cut into four equal sized pieces and immediately placed in 5ml of RNA stabilizing agent, RNA*later*™ (Ambion®). RNA*later*™ is a stabilizing solution that permeates tissues and cells and protects contained mRNA from degradation. The tissues were then stored at 4°C overnight, removed from the RNA*later*™ and stored at minus 80°C until RNA extraction.

2.2.3 Pre-term ovine foetal skin and spleen

Spleens and thoracic-flank-skin strips were collected from foetuses carried by date-mated experimental ewes. Access to foetal tissues was courtesy of Allan McNeilly, University of Edinburgh. The ewes were euthanised with intravenous pentobarbitone sodium and the intact gravid uterus removed to sample foetal tissues. The foetuses

were estimated gestational age (EGA) 60 (n=5), 70(n=5), 80(n=5) and 90(n=2) days (All EGAs being of second trimester pregnancy - ovine normal gestation 145 – 150 days). All collected samples were immediately placed in five times their volume of RNA*later*™ and stored overnight at 4ºC. After overnight storage, the RNA*later*™ was removed and the treated tissue samples stored at minus 80ºC until RNA extraction.

2.2.4 Steady state dendritic cells and PBMCs

2.2.4.1 Lymph fluid collection via canulation

Lymph fluid was collected from healthy sheep via surgically implanted canulae placed into a pseudo afferent lymph duct of the pre-femoral lymph node (Hopkins *et al.*, 1986). Pseudo afferent ducts were formed by surgically removing the pre-femoral lymph node several months prior to canula implantation for lymph collection. One week post-operatively, lymph was collected daily in previously autoclaved plastic bottles containing canulation fluid (containing actinomycin D ((Sigma) 1µg/ml), heparin (400U), penicillin (1000U)/streptomycin(1mg)). Dendritic cells were purified (Section 2.2.4.3.1) within twenty four hours of lymph collection. This system allows for the *in vivo* manipulation of DCs for *ex vivo* examination and the actinomycin D added to the collection fluid prevents *de novo* RNA synthesis.

2.2.4.2 Blood collection

Blood (40ml) was collected into heparinized tubes from healthy sheep donors via jugular venipuncture. PBMCs were extracted within four hours of blood collection.

2.2.4.3 Cell enrichment/extraction

2.2.4.3.1 Dendritic cells

Afferent Lymphatic DCs were extracted from lymph by centrifugation on a Ficoll-Metrizoate density gradient (density = 1.320; OptiPrep®; Axis-Shield); using the OptiPrep® at room temperature. Afferent Lymphatic cells were pelleted by centrifugation at 4°C, 300xg for 5 minutes. The cell pellet was washed once with ice-cold buffer B (see appendix II) and re-suspended in buffer B up to a maximum concentration of 2 x 10^7 cells/ml. 2.5ml of cell suspension was transferred into 20ml sterile universal containers and 1ml of OptiPrep® added and mixed gently. To this mixture, 4ml of freshly prepared OptiPrep® solution 1 (See appendix II for constituents; OptiPrep® solution1 densisty:1.078g/ml - lymphocyte specific density layer) was carefully overlaid, followed by 10ml of OptiPrep® solution 2 (See appendix II for constituents; OptiPrep® soluition2 density: 1.068g/ml) and finally overlaid with 1ml sPBS.

Gradient separation of DCs was achieved by centrifugation of this mixture at 600 x g for 25 minutes at room temperature, with the brake de-activated during deceleration at the end of the spin. Dendritic cells, in the gradient fraction above the OptiPrep® solution 1, were collected and transferred into a new 20ml sterile universal container, washed with sPBS and cells pelleted by centrifugation at room temperature, 300 x g, 5 minutes. The cells were re-suspended in ice-cold FACS buffer and centrifuged at 4°C, 300 x g, for 5 minutes. The supernatant was carefully decanted and the DCs re-suspended in 10 ml ice-cold FACS buffer, then counted and assessed for viability.

2.2.4.3.2 PBMCs

For PBMC extraction, 9ml histopaque® was pipetted into a standard 20ml sterile universal container. Onto this histopaque®, 10ml of an equal mixture of anticoagulant treated blood and PBS was carefully overlaid using an automatic pipettor set at the slowest speed (see Figure 2.2). The layered histopaque®/blood&PBS was centrifuged at 800 x g, 20 minutes at room temperature, with the brake at the end of the spin switched off. The PBMCs in the

interface were carefully removed using a Pasteur pipette and washed once with ice-cold sPBS; 300 x g, 5 minutes at 4°C. The cells were then counted and assessed for viability (see Appendix III for detailed protocol).

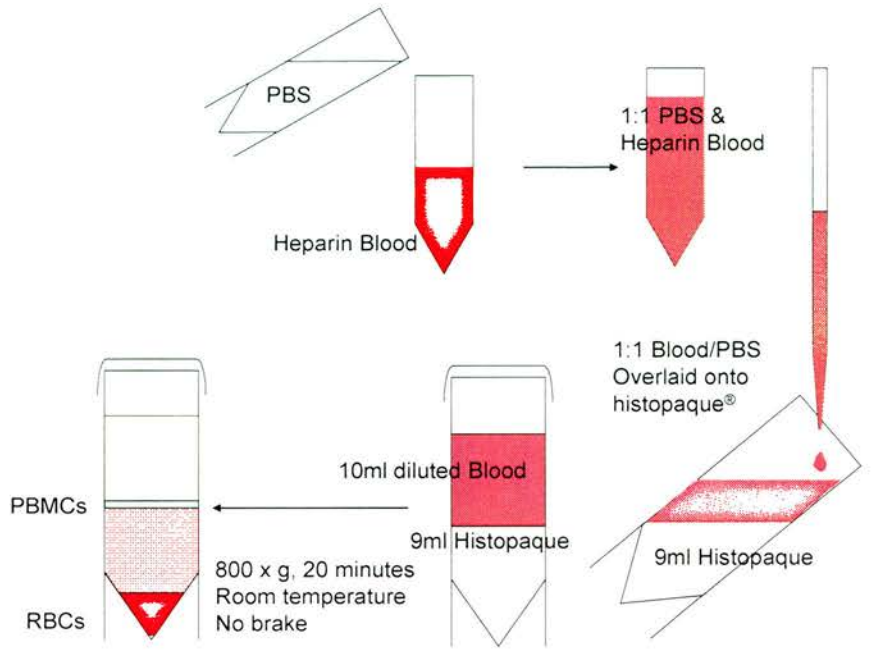


Figure 2.2 Schematic overview of the Histopaque® protocol for extraction of PBMCs.

Schematic overview of the histopaque® PBMC extraction protocol. Anti-coagulated blood is mixed with and equivalent volume of sPBS and mixed gently but thoroughly. 10ml of this mixture is carefully overlaid over 9ml histopaque® and the resultant mixture spun at 800 x g for 20 minutes at room temperature. PBMCs are aspirated with a Pasteur pipette at the interface.

2.2.4.4 Counting cells

Cells resulting from the cell enrichments were checked for viability using trypan blue exclusion. A 10µl volume of cell suspension was added to 10µl of 0.5% (w/v) trypan blue in PBS and mixed by gentle pipetting. A 10µl aliquot of the mixture was placed on a previously assembled Improved Neubauer haemocytometer. Cells were counted using a light microscope and x 200 magnification. The percentage viable cells was determined by counting cells that excluded the dye (viable cells) and those that took on the blue dye. The cells were then re-suspended to an appropriate cell concentration in FACS buffer for immunostaining.

2.2.4.5 Immunostaining and cell sorting

2.2.4.5.1 Flow cytometry

Isolated dendritic cells were double-stained with IL-A24 (anti CD172a) and biotinylated SW73.2 primary monoclonal antibodies conjugated to the following fluorochromes; anti-mouse Ig fluorescein isothiocyanate (FITC(Sigma)), and streptavidin phycoerythrin (SA-PE (Sigma)) respectively. PBMCs were incubated with the following antibodies, biotinylated STU-4 (conjugated to SA-PE), STU-T8, VPM30, andVPM65 (conjugated to FITC) (see Table 2.1).These cells were then subjected to fluorescence activated cell sorter (FACS) analysis using a *FACS Calibur*® or *FACS Scan*® cytometer (Becton-Dickson). FACS data were obtained by collecting a total of 10,000 cells (events) from each sample. Cells were acquired and gated based on the cells' expected characteristic forward scatter (FSC) and side scatter (SSC) profile. Different cell types have different profiles with dendritic cells having a high FSC and SSC representing a large cell size and complexity respectively (Figure 2.3). Appropriate compensation settings were determined for two-colour dendritic cell FACS analysis to overcome the overlap between the FITC and SA-PE fluorochrome spectrum. The analysis of the cell populations was done using the Cell Quest® software (Becton-Dickson). Post-acquisition screening data analysis and data presentation was also done using WinMDI software (http://facs.scripps.edu/software.html).

DC populations were purified using FACS (FACS*vantage*®, (Becton Dickinson)) at the University of Edinburgh Medical School, Inflammation Research Unit by Shonna Johnston. PBMCs populations were purified using FACS (FACS *Aria*®, (Becton Dickinson)) at University of Edinburgh, Ashworth Building by Andrew Sanderson.

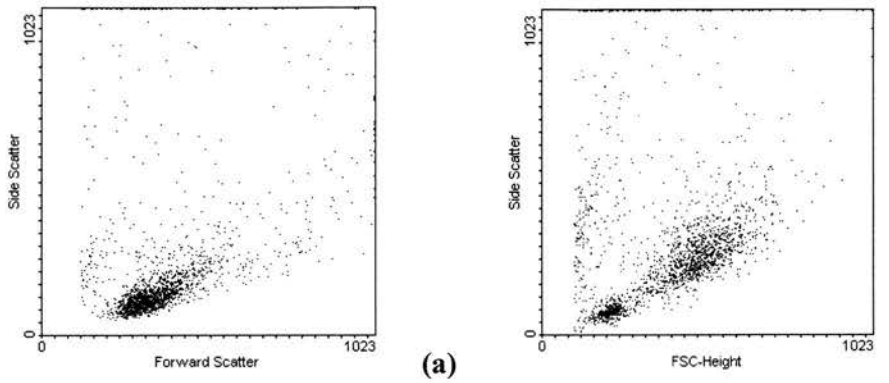


Figure 2.3 Dotplot of Unstained PBMCs and dendritic cells

Dotplot of a) Unstained Histopaque extracted PBMCs and b) unstained Optiprep® separated dendritic cells. Data displayed using WinMDI software. The PBMC dotplot (a) shows cells of relative homogeneity in terms of size and granularity as reflected by the tight distribution of the FSC and SSC profiles. Dendritic cells on the other hand have a greater heterogeneity in terms of size and granularity as reflected by their diverse FSC and SCC profiles from the dotplot (b). DCs are large and extremely granular cells.

2.2.4.5.2 Monoclonal antibodies

Monoclonal antibodies (mAb) used in this study are listed in Table 2.1. These mAb were obtained from stocks currently held within our Lab. Biotinylation of the SW73.2 was also performed in order to facilitate double immuno staining of dendritic cells.

mAb	Antigen	Reactivity	Target	Reference
IL-A24	Bovine SIRPα (CD172a)	Dendritic cells	DCs	(Ellis *et al.*, 1988)
SW73.2	MHC class II	B cells, activated T-cell, Dendritic cells	PBMCs DCs	(Hopkins *et al.*, 1986)
SBU-T8	CD8	T cells	PBMCs	(Maddox *et al.*, 1985)
SBU-T4	CD4	T cells	PBMCs	(Maddox *et al.*, 1985)
VPM30	28 kD	B-cells	PBMCs	(Naessens and Howard, 1991)
VPM65	CD14	Monocytes/ macrophages	Monocytes/ macrophages	(Gupta *et al.*, 1996)

Table 2.1 Monoclonal antibodies (mAb) used for immunostaining and FACS analysis

Monoclonal antibodies (mAb) used for immunostaining cells for FACS analysis. Target cell type and original references listed. ILA-24 and SW73.2 were used for double staining dendritic cells and the rest of the antibody panel was used for single staining peripheral blood mononuclear cells.

2.2.4.6 Immunostaining

2.2.4.6.1 One colour immunostaining

Single colour immunostaining was carried out to allow FACS sorting of PBMC populations. During immunostaining, all procedures were done on ice, and working as quickly as possible. Following histopaque® extraction, PBMCs were counted and re-suspended in ice-cold FACS buffer to a concentration of 2 x 10^8cells/ml and 50µl dispensed into FACS tubes (Falcon). The cells were first incubated with an equal

volume of 1:500 diluted Normal Mouse Serum (NMS) for 15 minutes at 4°C. To remove unbound NMS, the cells were washed twice with 2.5ml FACS buffer/FACS tube and centrifugated at 4°C, 300 x g, for 5 minutes. The cells were then incubated for 15 minutes at 4°C with an appropriate amount of monoclonal antibody (Table 2.1) based on concentrations derived from individual mAb titration (Section 2.2.4.6.3). To remove unbound mAb, the cells were washed twice with 2.5ml FACS buffer/FACS tube and centrifugation at 4°C, 300 x g, for 5 minutes. For the biotinylated STU-T4, the cells were incubated with streptavidin phycoerythrin (SA-PE (Sigma)) 5µl/1 x 10^6 cells and incubated for 15 minutes at 4°C. The non-biotinylated mAb were detected following similar incubation but using 50µl anti mouse Ig FITC (Sigma)) diluted 1:80. The cells were again washed twice. Cells for sorting were then re-suspended in ice-cold FACS buffer at a concentration of not more than 5 x 10^6 cells/ml and sorting carried out immediately. Cells that were to be used for FACS analysis were re-suspended in 2% (w/v) paraformaldehyde in PBS and stored at 4°C, covered in aluminium foil until use. FACS analysis of paraformaldehyde stored cells was normally done within 48 hours (see Appendix III).

2.2.4.6.2 Two colour immunostaining

Two colour immunostaining was carried out to facilitate FACS sorting of $CD172a^+$/ $CD172a^-$ DCs populations. The cells were incubated with the ILA-24 and SW73.2 mAb for 15 minutes at 4°C. NMS blocking and washing were as for the single colour immunostaining above.

2.2.4.6.3 Titration of monoclonal antibodies

Prior to being used for immunostaining for FACS cell sorting, respective mAb used in these experiments were titrated on extracted PBMCs and DCs to establish optimum working concentrations. An optimum volume of supernatant (or dilution for concentrated biotinylated mAb) of the primary antibody, i.e. that which gave strongest fluorescence signal and lowest background staining, was determined for each mAb. Where appropriate the anti mouse Ig FITC dilution was adjusted from the standard 1:40 dilution by titration.

2.2.5 Ovine paratuberculosis tissue collection

A retrospective study of ovine ileum tissues was carried out from sheep flocks naturally infected with paratuberculosis. Archival distal ileum samples, stored in RNA*later*™ (Ambion), were from 24 cases of Johne's disease diagnosed at Moredun Research Institute's veterinary postmortem service. All cases had clinical histories, typical of Johne's disease and all cases were confirmed by gross pathological changes at necropsy, culture and histopathology. There were 8 cases each of; paucibacillary, mutlibacillary and 'asymptomatic' designation (Appendix V). Access to these tissues was courtesy of Dr. Craig Watkins (Mycobacterial Research Group, Mordeun Research Institute, Edinburgh) and RNA was extracted by Jennifer Smeed. 'Normal' ileum tissue was also collected from sheep at the Marshall building, Roslin; a facility that has had a low incidence of Johne's disease (Docherty, personal communication).

2.2.6 PRR expression in ligand stimulated ovine skin

2.2.6.1 Preliminary study (dosage test)

An initial pilot experiment was undertaken to determine an appropriate concentration of *Mycobacterium avium paratuberculosis* strain V lipoarabinomannan (LaM) to use for carrying out a cutaneous LaM stimulation experiment to determine resulting innate immune responses via PRRs. Three concentration of the LaM were used for this pilot study namely, 100μg/ml, 200μg/ml, 400μg/ml. 100μl of each concentration of the ligand were administered to one animal as outlined in Section 2.2.6.3. The LaM was administered in duplicates and at time points 8hr, 4hr, 2hr and 1 hour prior to euthanasia.

2.2.6.2 Study animals

For the experiment, four, one-year old Boorola male sheep were administered with 50µl (50µg/ml) LaM as outlined in Section 2.2.6.3.

2.2.6.3 Ligand administration

The flank of the study sheep was shaved with electric clippers covering the area from the *tuber coxae* to the 10th rib caudo-cranially and a distance of approximately 30 cm from the level of the transverse processes ventrally. The clipped area was washed with warm water and de-fatted with 100% ethanol followed by an iodine scrub and wash. The flank was marked with grids using an indelible marker representing the intradermal injection sites and time points (Figure 2.4).

In order to ease the discomfort to the study subjects, the ligand solutions were warmed to approximately body temperature by holding the vials in a clasped hand for about a minute prior to intradermal injections. 50µl LaM or sPBS were administered in triplicates 24hr, 8hr, 4hr 2hr and 1 hr prior to euthanasia and skin biopsy collection. Euthanasia was carried out using 200mg/ml intravenous pentobarbitone sodium. Biopsies were collected using 6mm disposable biopsy punches and excessive fat and connective tissue were trimmed off. Half of one biopsy was placed into 5ml zinc sulphate fixative solution (Gonzalez *et al.*, 2001) (Appendix VI) for histopathology. All other collected biopsies were immediately placed in RNA*later*™ and stored at 4°C overnight, then removed from the RNA*later*™ and stored at minus 80°C until RNA extraction. The skin biopsy in the fixative was also stored at 4°C overnight, and the fixative replenished with double the volume fresh fixative. Within 48 hours post-mortem, the biopsy samples were sent to Easter Bush Veterinary Centre (University of Edinburgh (EBVC), Midlothian) for automated processing paraffin wax embedding, sectioning and Haematoxylin and eosin staining.

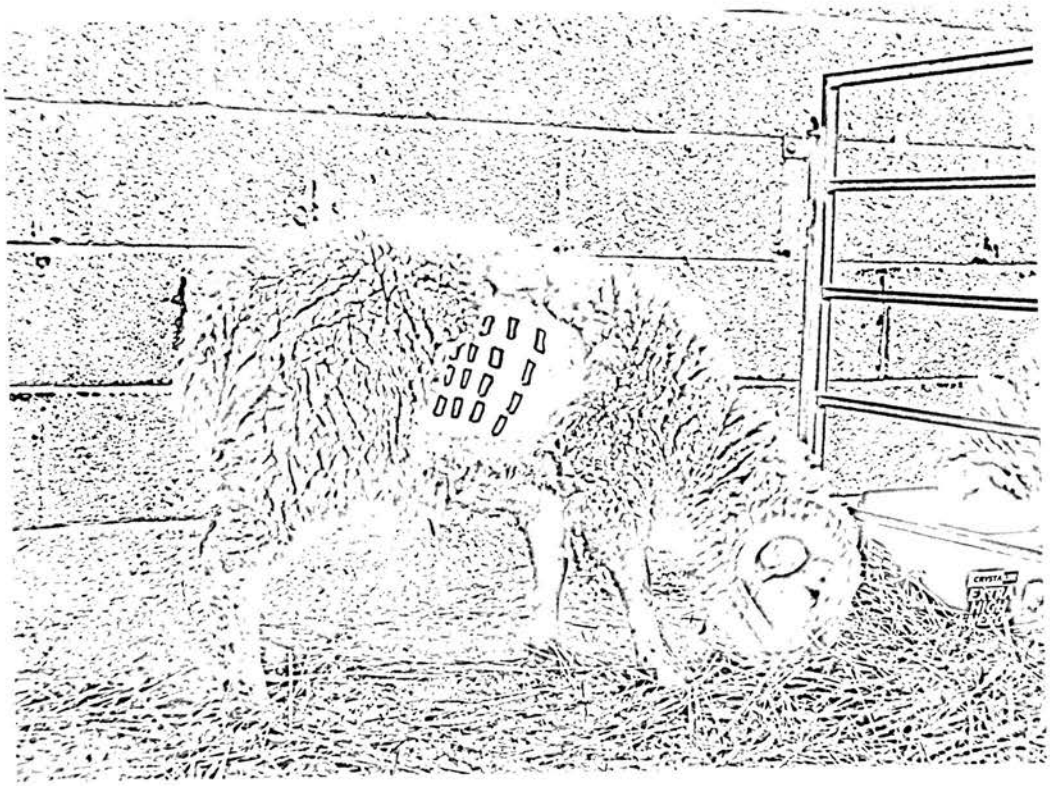


Figure 2.4 Sheep prepared for cutaneous ligand administration.

Figure showing the shaved area of the right flank of a study sheep and a permanently marked grid representing the location for intradermal injections. Each box was at least 4cm x 2cm in dimension and boxes were at least 2cm apart. Two intradermal injections were administered into each box at the polar ends of the rectangles and the middle of the intradermal bleb marked with a marker pen tip to facilitate accurate biopsy punching (post mortem).

2.3 Nucleic acid extraction

2.3.1 Good molecular biology practices

Most methods used in this study for DNA or RNA manipulation were based on methods in 'Molecular Cloning: A Laboratory manual' (Sambrook *et al.*, 1989) and the Laboratory kits' manufacturer's guidelines. Prior to use, all bench-top surfaces, equipment and pipettors were decontaminated using 10% Decon®, followed by 70% ethanol and finally with RNAse*Away*® and Microsol[3]® to avoid contamination. Nuclease free-sterile plastic ware and aerosol resistant filter tips were utilized and disposable gloves were changed frequently whilst carrying out all the molecular biological procedures.

2.3.2 RNA extraction

2.3.2.1 Cells and normal tissues

RNA was extracted from tissues and cell types collected from healthy sheep populations. The RNA extraction process from sorted PBMCs and DCs was begun on the same day of cell isolation. Cells purified as in section 2.2.4.3 were re-suspended in RLT buffer with 1% β-mercaptoethanol, vortexed briefly and the cell lysate stored at -80 °C until completion of the RNA extraction process. RNA was extracted using commercial RNA extraction kits - RNeasy® Midi Kit and Mini Kit (Qiagen) following the manufacturer's protocol.

2.3.2.2 Ligand stimulated skin

An approximately 25mg piece of RNA*later*™ preserved biopsy sample was placed onto an individual, sterile, disposable Petri dish and cut up into small pieces. The pieces were then carefully placed into a clean RNAse free, 1.5ml microcentrifuge tube tube into which 200µl of lysis buffer (RLT with β-mercaptoethanol) was added. In order to get a uniformly homogenized sample, skin was homogenized using a motorized pestle. Freshly autoclaved individually packed pestles were used for each sample. Half way through the homogenization process, another 150µl of the lysis

buffer was added. After obtaining a homogenate of uniform consistency with no tissue clumps, 550µl of nuclease free water was added and mixed well. To the mixture, 10µl of proteinase K (Sigma; 20mg/ml) was added and mixed well by light vortexing. The suspension was incubated in a water-filled hot block at 55°C for fifteen minutes. After incubation, genomic DNA was sheared using an RNAse free syringe and 20G needle and passing the lysate through the needle at least five times.

The homogenate was subsequently centrifuged at 10,000 x g for 3 minutes and the supernatant collected and placed into a clean 1.5ml microcentrifuge tube. An equal volume of 70% ethanol was added to the supernatant and mixed well prior to dispensing onto the Qiagen RNeasy mini spin columns. All subsequent steps were according to the manufacturer's protocol and final elution was carried out in 30µl of RNAse free water.

2.3.2.3 Removal of trace DNA contamination – DNAse I treatment

In order to remove trace quantities of contaminating DNA, DNAseI treatment of eluted RNA was carried out. Briefly, 1/19th of the eluate volume of 20x DNAse buffer was added to the RNA followed by 1µl of the DNAseI enzyme (8 units/µl). After gentle mixing, the mixture was incubated at 37°C for thirty minutes. This was followed by a DNAse deactivation stage at room temperature. One fifth of the total eluate volume of DNaseI inactivation slurry was added to the mixture and stored at room temperature for two minutes. After the two minute incubation, during which the slurry mixture was mixed gently and constantly, the mixture was centrifuged at 10,000 x g for 1 minute to pellet the deactivation agent. The DNA free RNA containing supernatant was collected by pipetting and placed into an appropriately labelled, clean 1.5ml microcentrifuge tube. From the total sample, one 5µl aliquot of RNA was removed for spectrophotometry and a 2µl aliquot for RNA integrity analysis with the Agilent® 2100 bioanalyzer. All RNA samples and aliquots were stored at minus 80°C until use.

2.3.2.4 Sample integrity confirmation.

Total RNA was quantified using spectrophotometry - using the formula that $OD_{260\ nm}$ (absorbance at wavelength $OD_{260\ nm}$) of 1.0 = 40μg/ml RNA. As a measure of purity the ratio of the absorbance at 260nm/absorbance 280nm was determined, with pure RNA free of protein contamination having a ratio of 2.0. The RNA quality and integrity was further confirmed using the Agilent® 2100 bioanalyzer. Ideal RNA has an electropherogram showing rRNA Ratio [28s/18s] approaching 2.0 and an RNA integrity number (RIN) close to 10.0 as shown in Figure 2.5. RNA samples with rRNA ratios greater than 1.4 and RIN greater than 7.0 were considered acceptable for use.

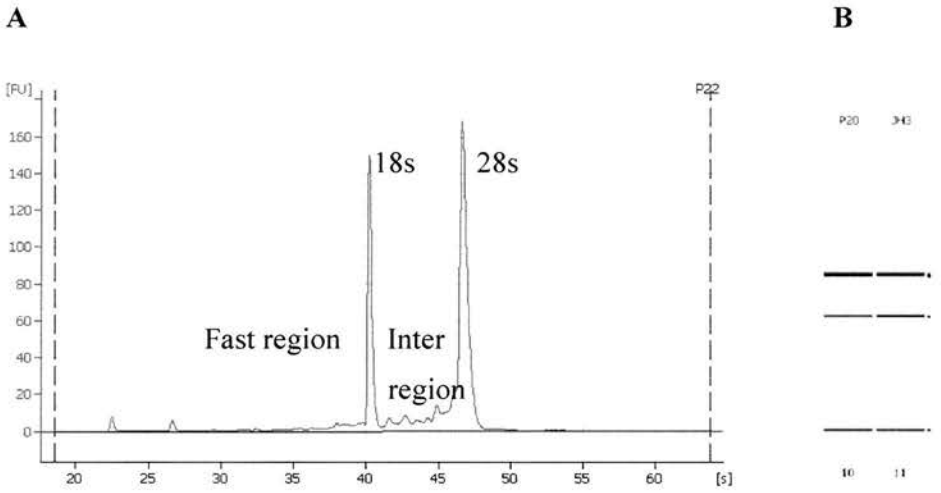


Figure 2.5 Agilent 2100 Bioanalyzer Electropherogram and gel view

A) Segment of an electropherogram showing the 18s rRNA peak (left shorter peak) and the 28s rRNA peak (right higher peak) with minimal peaks in the fast region and inter region. The 28s peak is higher with a larger area under it than the 18s region indicating good quality RNA with a high 28s/18s. **B)** A sample Agilent 2100 bioanalyzer gel view of good quality RNA showing two distinct bands representing 18s and 28s rRNA peaks.

2.3.3 DNA extraction

Genomic DNA (gDNA) was extracted using a commercial kit, DNeasy® (Qiagen). Briefly, 20mg of tissue was placed on a sterile clean Petri dish and chopped up into small pieces. The pieces were transferred into a clean 1.5ml microcentrifuge tube tube into which 20µl proteinase K was added and mixed. The mixture was incubated at 55°C until the tissue was completely lysed. The mixture was vortexed for 15s, 200µl buffer AL added, mixed and incubated at 70°C for 10 minutes. The mixture was vortexed again prior to, and after the addition of 200µl pure ethanol. This mixture was transferred into a DNeasy® spin column and washing with the manufacturer's buffer AW1 and AW2 carried out according to the manufacturer's protocol followed by an elution with 150µl buffer AE. The DNA was quantified using a spectrophotometer and stored at minus 20°C until use.

2.4 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

2.4.1 First strand cDNA synthesis – Reverse transcription

RNA was reverse transcribed using Oligo(dT)$_{15}$ primers and using either Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega) for the tissues or Omniscript® (Qiagen) for the cells. The cDNA was used for all subsequent conventional PCRs and quantitative real time PCRs.

2.4.1.1 cDNA synthesis - tissues

For each RNA sample, cDNA was synthesized using M-MLV reverse transcriptase kit (Promega). For the conversion of RNA into cDNA, reverse transcription (RT) was done using 0.5μg Oligo(dT)$_{15}$ primers and reactions were carried out in a final volume of 25 μl. For the first strand cDNA synthesis, 2.5μg of total RNA, together with the 0.5μg Oligo(dT)$_{15}$ primers was heated in a thin-walled PCR tube at 70 °C for 5 minutes and cooled quickly on ice for a further 5 minutes. To the annealed primer template mixture 5μl of M-MLV RT 5X reaction buffer, 1μl of dNTPs mix (10mM), 1μl RT enzyme and nuclease free water up to 25μl were added. After gentle mixing, the reaction was incubated at 40°C for 10 minutes, 42°C for 50 minutes and the reaction inactivated at 70°C for 15 minutes. The cDNA was diluted four-fold in nuclease free water and stored at -20°C until use. For the initial PRRs identification experiments the cDNA was used neat.

In order to avoid discrepancy arising during cDNA synthesis, sufficient cDNA synthesis from each tissue set was carried out in a single batch to avoid any likely variability caused by differences in the reverse transcription efficiency of target and reference gene. A second batch of cDNA synthesis was performed and the two batches mixed.

2.4.1.2 cDNA synthesis – FACS sorted cells

Reverse transcription of RNA derived from cell subsets was carried out using Omniscript® Kit (Qiagen). This kit is designed for optimal cDNA synthesis from low quantities of RNA template (50ng - 2µg). RT was carried out using 200ng RNA and a final volume of 20µl. All pre-incubation steps in this protocol were carried out on ice. A master mix was prepared using the supplied reagents comprising; 2µl 10x buffer, 2µl 5mM dNTPs, 2µl Oligo$(dT)_{15}$, 1µl (10 units/µl) RNase inhibitor and 1µl Omniscript®. Reverse transcriptase for each RT reaction. Into each labelled, thin-walled PCR tube 8µl of the master mix was added, followed by template RNA and made up to 20µl with ice-cold nuclease free water. The tubes were mixed by light vortexing and the contents collected by pulse centrifugation. The mixtures were incubated at 37°C for 1hour. The cDNA was diluted two-fold in nuclease free water and stored at -20°C until use.

2.4.2 Primers

Primers used in this work are shown in Table 2.2. The sequences of sheep PRRs were not available at the commencement of the study; therefore primers were designed using bovine PRR sequences deposited in Genbank or The Institute of Genomic Research (TIGR). Human mouse consensus GAP sequences were used for the design of TLR5 and TLR7 primers. The partial bovine sequence for TLR8 was kindly provided by Dr. EJ Glass, Roslin Institute, Edinburgh.

The primers were selected and designed using the Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) software, a web based program. Where intron-exon structures of the genes were known, primers were designed to span an intron(s) or intron/exon splice junction to obviate the possibility of amplification of genomic DNA. All selected primer sequences were then checked for possible cross-hybridization by subjecting them to a BLAST (http://www.ncbi.nlm.nih.gov/BLAST/index.shtml) database similarity search program (Altschul *et al.*, 1990). The primers were then subjected to quality check using Premier Biosoft (http://www.premierbiosoft.com/netprimer/index.html). All primers were obtained from Sigma-Genosys Ltd, UK.

Table 2.2 Primers used in the Study

Primer Name and sequence	Annealing temp	Product size	Source Accession#
Toll-like receptor 1			
TLR1 For 5`- GAATTTCTGGGGTTGAGTGC TLR1 Rev 5`-CTGGAGGATCGTGAAGAAGG	58	375 bp	AY634628
Toll-like receptor 2			
TLR2 For 5`- TGCCTGAAACTTGTCAGTGG TLR2 Rev 5`-AATGGCCTTCTTGTGTCAATGG	58	953 bp	AY634629
*Toll-like receptor 3****			
TLR3 For 5`- GGGTCTGGAAGCACTTCTCC TLR3 Rev 5`- GCTGAATTTCTGGACCCAAG	59	403 bp	AY124007
Toll-like receptor 4			
TLR4 For 5`-GGACAACCAACCTGAAGCAT TLR4 Rev 5`-CCCTGTAGTGAAGGCAGAGC	58	945 bp	AF310952
Toll-like receptor 5			
TLR5 For 5`- CCAGAGTCTGCTGTTCAAGG TLR5 Rev 5`-ACCCTCTGATGGACTGATGC	57	404 bp	AB060695 & AF186107
Toll-like receptor 6			
TLR6 For 5`- TTTGTCCTCAGGAACCAAGC TLR6 Rev 5`- TGGGCTAAAGAATTGGAAGC	58	421 bp	AJ618974
Toll-like receptor 7			
TLR7 For 5`- ATGCTGTGTGGTTTGTCTGG TLR7 Rev 5`- GGTCACGTGATTGTCTGTGG	58	752 bp	AF245702 & AY035889
Toll-like receptor 8			
TLR8 For 5`- GGTTTTACTGGGATGCTTGG TLR8-Rev 5`-TCTCCTCCATTAGCCTCTGC	57	358 bp	

N.B. Table of primers used continued on the following page.

↑ yours?

Table 2.2 Primers used in the Study (continued)

Primer Name and sequence	Annealing temp	Product size	Source Accession#
Toll-like receptor 9			
TLR9 For 5`- GACCTGTCACGGAACAACCT TLR9 Rev 5`- GGAGGGATCCACTGTCTTCA	58	798 bp	AJ509825
Toll-like receptor 10			
TLR10 For 5`- ATACTTGGAGTGGCCCAAGG TLR10 Rev 5`- ACACATGGGAAACCAACTGC	58	648 bp	DQ058827
Beta actin			
β-actin For 5'- ACTGGGACGACATGGAGAG β-actin Rev5'- AGGAAGGAAGGCTGGAAGAG	58	538 bp	U39357
MyD88			
MyD88 For 5'- CCTGTCGCTCTTCCTAAACG MyD88 Rev 5'- CAATGAGTTCACTGGCGATG	58	542 bp	AJ853453
CD14			
CD14 For 5'- CGGGTACTCTCGTCTCAAGG CD14 Rev 5'- AGATCATCGGGTCATTTTGG	56	695 bp	D84509
*CARD15 (NOD2)****			
CARD15 For 5' - ACTCTGCCTGGAGGAGAACC CARD15 Rev 5'- AGTGGACGAACCACTCAACC	58	621 bp	AY518748
*Dectin-1*** α- isoform*			
Dectin-1 For 5' - CTCATTGGCGTCTGATTGC Dectin-1 Rev 5' - CCATGGTTCTTCTGTCTGACG	61	455 bp	AY937383
*Dectin-2***α- isoform*			
Dectin-2 For 5' - CCTCCTCAGTGCATGTTTCA Dectin-2 Rev 5' - GGAATATCCCCATGACCTGA	61	658 bp	DQ176046
Succinate dehydrogenase (SDHA) **			
SDHA For 5' - ACCTGATGCTTTGTGCTCTGC SDHA Rev 5' - CCTGGATGGGCTTGGAGTAA	59	126 bp	NM174178
*GAPDH***			
GAPDH For 5' - AAGGCAGAGAACGGGAAG GAPDH Rev 5' - AGT GAT GGC GTG GAC AGT	55	865 bp	AF022183

*** These primers span an intron(s) based on BLAST results of the bovine genome.

** These primers were kindly provided by other members of our Lab group.

2.4.3 Polymerase Chain Reaction (PCR)

PCR was carried out using a standard protocol described by Promega®, with modifications in reaction conditions to suit each primer pair. For each PCR run, positive and negative controls were included. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR was performed on all reaction sets as a positive control to confirm the presence of cDNA. Nuclease free water was used (in the place of the cDNA template) as a negative control.

For the PCR amplification 2µl of cDNA synthesized from 1 µg of total RNA from the RT reaction (section 2.4.1) was utilized and carried out in a final volume of 50µl. Primers used for each PRR are shown in Table 2.2 above. For multiple reactions, all reactants (except for the *Thermus aquaticus* (Taq) and template) were mixed together as a master mix (Table 2.3). For a Z reaction master mix (where Z is the total number of PCR reactions required), Z+1 of each reagent were aliquoted to take care of aliquoting variations. The master mix was prepared in a nuclease free microcentrifuge tube as outlined below and after thorough mixing; 47.75µl was aliquoted into each thin-walled PCR tube: The cDNA template was added to each tube prior to the 'hot start' denaturation stage and the Taq added after this stage.

Table 2.3 Preparation of a PCR 'master mix'

Reagent	**Each**	**Mix (Z+1) reactions**
10 x PCR buffer with 1.5mM $MgCl_2$ (Promega)	5µl	(Z + 1) x 5µl
dNTPs mix	1µl	(Z + 1) x 1µl
Primers (20pmol) Forward	0.2µl	(Z + 1) x 0.2µl
Primers (20pmol) Reverse	0.2µl	(Z + 1) x 0.2µl
Template (cDNA or Nuclease free water)	2µl	
Taq polymerase *(Thermus aqnauicus* enzyme*)*	0.25µl	
Nuclease free water (up to 50µl)	41.35µl	(Z + 1) x 41.35µl
Total vol.	**50µl**	**(Z + 1) x 47.75µl**

PCR amplification was carried out in a Px2 thermocycler (Thermo Electron Corporation) using the 'hot start' principle and a temperature gradient. Reaction conditions i.e. annealing temperature, Mg^{++} concentration and cycle conditions for each primer set were optimized. Manual 'hot start' was employed to reduce the chances of mispriming and non-specific amplification products. The PCR mixture (without the Taq polymerase) was initially denatured at 95 °C for 2 minutes prior to the addition of the Taq polymerase enzyme. The now complete reaction mixture was then cycled under the following general conditions: Thirty cycles of denaturing at 95°C, 60s; annealing at a temperature gradient 60s; and extension at 72°C, 60s. This was followed by a final extension at 72°C for 10 minutes. Amplicons were visualised by gel electrophoresis and ethidium bromide staining (section 2.5.1) to determine the optimum candidate annealing temperatures and if there was need to adjust Mg^{++} concentration.

2.5 PCR Product identification

2.5.1 Agarose Gel Electrophoresis

Resulting PCR products were size fractionated and analysed using ethidium bromide agarose gel electrophoresis to determine the quality and size of the amplicons. The percentage of the agarose in the gels used in this study varied from 0.7% to 2.5% (w/v) in TAE buffer depending on the expected size of the PCR product – a lower percentage for expected larger amplicons and a higher percentage for expected smaller amplicons, see appendix VII. Gels were made in 70ml TAE buffer (20mM Tris, 1mM EDTA, 0.1% (v/v) glacial acetic acid). The agarose in TAE buffer was mixed well and heated in a microwave oven for 90 seconds until the agarose was completely dissolved. The agarose was allowed to cool to about 60°C and 0.5µl ethidium bromide (10µg/ml) added and mixed well. The melted agarose was poured into a tray with an appropriate comb and the gel was allowed to set for at least 30 minutes prior to loading.

Five to ten microliters of sample was mixed with 2µl of blue/orange DNA loading dye (Promega®) and added to each well. The first and last well contained 100bp ladder (Promega®) to allow for size determination. All gels undergoing electrophoresis were subjected to 80 - 100V steady voltage for 45 to 80 minutes depending on speed of dye migration.

After electrophoresis, the gel was placed on a UV light trans-illuminator for visualization, and the fluorescent ethidium bromide stained DNA pattern captured with a camera for image storage and printing. Fragment size was determined by comparing with a 100 base pair and 1 kilo base pair DNA ladder (Promega®).

Once the expected size of amplicons was confirmed, remaining PCR product in the PCR tube was purified for restriction mapping and eventual cloning and sequencing.

2.5.2 PCR Product Purification

PCR products were purified using the QIAquick® kit (Qiagen). Briefly, five volumes of binding buffer (PB) were added to the PCR reaction and mixed well by pipetting. The mixture was then added onto the QIAquick® spin columns and centrifuged in a desktop centrifuge at 13,000 rpm for one minute. The flow through was discarded and the column washed by placing 750µl of the manufacturer's wash buffer PE onto the column and centrifuging for one minute at 13,000 rpm. After discarding the flow through, the spin column was placed in a new 1.5ml microcentrifuge tube and centrifuged again for one minute at 13,000 rpm to remove any excess ethanol. The column was placed into a fresh nuclease free 1.5ml microcentrifuge tube and cDNA was eluted by placing 50µl of the manufacturer's elution buffer (EB) on the centre of the column, incubating at RT for at 1 minute and centrifuging at 13,000 rpm for one minute.

2.5.3 Gel extraction PCR amplicon

Where multiple bands of PCR products were present, the amplicon of interest was purified using a QIAquick® gel extraction kit (Qiagen). Briefly, working as quickly as possible, under the UV transilluminator and taking all safety precautions; the DNA band of interest from the agarose gel was excised with a new sterile scalpel. The gel slice was placed into a previously weighed colourless 1.5ml microcentrifuge tube and the weight of the gel determined. Three volumes of the manufacturer's buffer QG were added to one volume of gel (1mg ~ 1 µl, no more than 400mg of gel). The mixture was incubated at 50°C and vortexed often until the gel was completely dissolved. One gel volume of isopropanol was added and mixed by inverting several times. The mixture was transferred into a QIAquick® spin column and centrifuged at room temperature, 13,000 rpm for one minute. The flow-through was discarded and the column washed with 750 µl of the manufacturer's buffer PE and centrifuged twice at 13,000 rpm. The column was transferred into a clean 1.5ml microcentrifuge tube and DNA eluted with 50 µl of the manufacturer's buffer EB.

2.5.4 Restriction mapping - Restriction enzyme digestion

Once the expected size of product was obtained, the initial verification of sequence of the PCR product was achieved using restriction digests of purified PCR product. Restriction maps were generated, to determine appropriate restriction endonucleases, using the bovine sequence of amplicon and the web-based HGMP Jemboss® remap® program (http://www.hgmp.mrc.ac.uk/Software/EMBOSS/Jemboss/) and another web based software, NEBcutter® V2.0 (http://tools.neb.com/NEBcutter2/index.php). Single cutting enzymes resulting in markedly differing fragment sizes were preferred. Each of the enzymes chosen was specific to a particular restriction site contained within the expected amplicon. 0.5-2 units of enzyme were used for 0.2 - 2.0μg of cDNA per reaction and incubation times of 1 hour to 3 hours at 37°C. The list of restriction enzymes used for the different PRRs is shown in Table 2.4.

After restriction digests, the digest was subjected to ethidium bromide gel electrophoresis for verification of restriction fragments sizes. In cases where the first digests failed alternative enzymes, cutting elsewhere on the amplicon sequence, were utilized, to take care of the possibility of slight differences in the actual ovine and source bovine sequences used to generate the restriction map.

Restriction map verified PCR products were cloned using a commercial kit from pGEM-T Easy® vector (Promega).

Table 2.4 Restriction enzymes used and resultant expected restriction map fragment sizes

Bovine gene	Restriction Enzyme	Restriction site sequence	Expected cut sites	Expected Fragment sizes
TLR 1	***HinfI***	5' G$^{\Downarrow}$ANTC 3' 3' CTNA$_{\Uparrow}$G 5'	1	219,156
TLR 2	***Pst I***	5'CTGCA$^{\Downarrow}$G3' 3'G$_{\Uparrow}$ACGTC5'	1	595/358
	Sal I	5'G$^{\Downarrow}$TCGAC3' 3'CAGCT$_{\Uparrow}$G5'	1	384/569
TLR 6	***EcoRI***	5' G$^{\Downarrow}$AATTC 3' 3' CTTAA$_{\Uparrow}$G 5'	1	197/224
	Hinf I	*As above for TLR1*	1	285/136
TLR 7	***EcoRV***	5' GAT$^{\Downarrow}$ATC 3' 3' CTA$_{\Uparrow}$TAG 5'	1	286/466
	RSA I	5' GT$^{\Downarrow}$AC 3' 3' CA$_{\Uparrow}$TG 5'	2	512/191/239
TLR 8	***AvaI***	5' C$^{\Downarrow}$YCGRG 3' 3'GRGCY$_{\Uparrow}$C 5'	1	215/14
TLR10	***PstI***	*As above for TLR2*	1	148/500
	XmnI	5'GAANN$^{\Downarrow}$NNTTC 3'CTTNN$_{\Uparrow}$NNAAG	1	310/338
CD14	***PstI***	5'CTGCA$^{\Downarrow}$G3' 3'G$_{\Uparrow}$ACGTC5'	1	155/540
	XmnI	*As above for TLR10*	1	319/376
	AvaI	*As above for TLR8*	2	82/157/456
Dectin-2	***DraIII***	5'CACNNN$^{\Downarrow}$GTG 3'GTG$_{\Uparrow}$NNNCAC	1	252/443
	HinfI	*As above for TLR1*	1	200/495
MyD88	***HinfI***	5' G$^{\Downarrow}$ANTC 3' 3' CTNA$_{\Uparrow}$G 5'	1	244/298
	XhoI	5'C$^{\Downarrow}$TCGAG 3' 3'GAGCT$_{\Uparrow}$C 5'	1	172/370
	AvaI	*As above for TLR8*	2	166/172/204

Key: N= Any nucleotide

2.5.5 PCR – Genomic DNA

For those primers designed to span introns (Primers for TLR3, CARD15, qTLR3 and qCARD15), PCR was performed on ovine genomic DNA to ascertain the absence of PCR product identical to cDNA derived amplicons.

2.6 Cloning and Sequencing of PRR Amplicons

Each of the PRR PCR amplicons were individually inserted into pGEM T-Easy® (Promega), and the recombinant vectors transformed into JM109 (Promega) high efficiency competent cells. The pGEM T-Easy vector system has a great ease of inserting PCR amplicons into the vector and this can be further confirmed by colour screening of bacterial clones grown on LB/ampicillin/IPTg/X-gal agar plates.

2.6.1 Ligation Reactions

The volume of PCR amplicon required to achieve 1:3 1:1 and 3:1 (vector: insert) molar ratios was calculated. Approximately 50ng of the pGEM-T Easy® vector (Promega) was ligated to QIAquick® (Promega) purified (Section 2.5.2) PRR amplicon at the appropriate molar ratio. Reactions were carried out in a total volume of 10µl, containing 1µl vector, 5µl 2X ligation buffer, 1µl T4 DNA ligase and made up to 10µl with water and insert. The reaction was mixed gently and incubated overnight at 4°C. Positive and negative controls were included to assess ligation efficiency according to manufacturer's recommendations.

2.6.2 Transformation of High Efficiency Competent Cells

All pipette tips and 15ml Falcon® tubes used during the transformation were stored at -20°C overnight prior to use and kept on ice whilst carrying out the transformation. Following overnight incubation, the ligation reaction was pulse centrifuged and 2µl added to 25µl of JM109 (Promega) High efficiency competent cells, mixed gently

and incubated on ice for 20 minutes. The cells were then heat-shocked for 50 seconds in a water bath at 42°C and then returned on ice again for 2 minutes. To these transformed cells, 975µl of SOC medium (Invitrogen) at room temperature was added and incubated at 37°C, with constant shaking, for 1.5 hours. Following this incubation, 100µl of transformed cells were plated onto an LB/ampicillin/IPTG/X-gal (1.5 % agar in Luria Bertani media supplemented with 50µg/ml ampicillin, 200mM IPTG (isopropyl thiogalactoside) and 0.04% X-gal (5-Bromo-4-Chloro-3-Idoly-β-D-Galactopyranoside)) agar plate and labelled *LOW*. The remaining cells were centrifuged at 2400 rpm and 500µl of supernatant removed. The remaining amount was flicked gently to re-suspend the cells and then 100µl of that was plated onto an LB/ampicillin/IPTG/X-gal agar plate as before and labelled *HIGH*.

These plates were incubated at 37°C overnight and then placed at 4°C to facilitate blue colour development of the colonies. Two single, isolated white colonies from each plate (high and low) were picked and inoculated into 10ml LB medium and incubated overnight at 37°C with constant shaking. These fresh overnight cultures were then used for plasmid DNA isolation.

Figure 2.6 pGEM-T Easy® Vector map and sequences with restriction sites

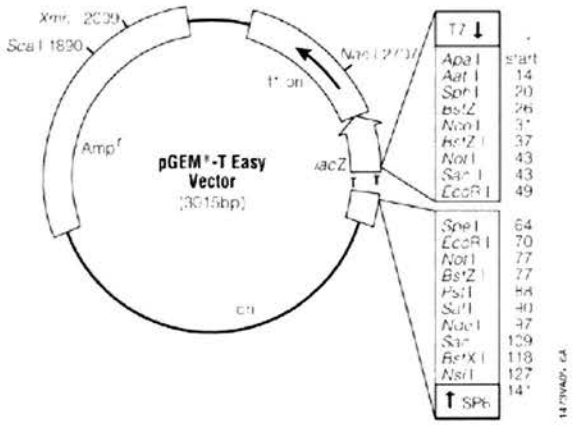


Figure 2.7 pGEM -T Easy® multiple cloning sites

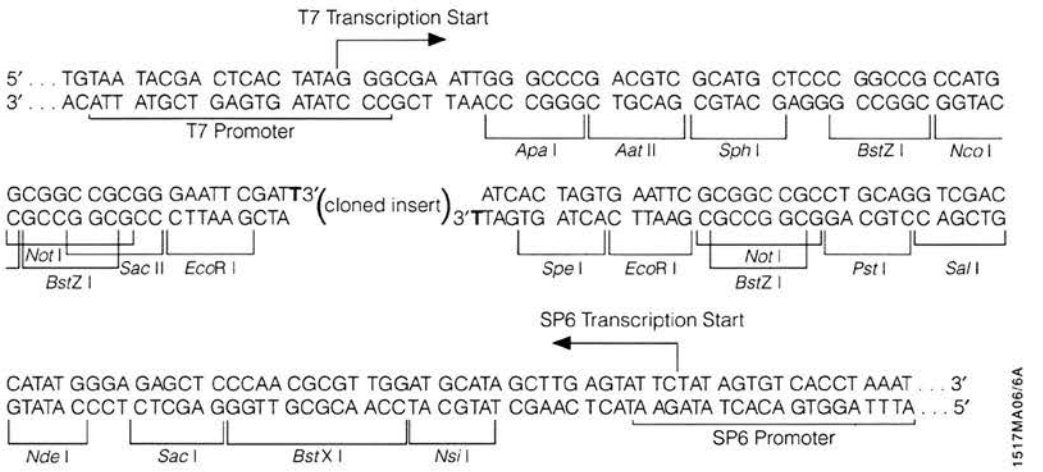


Figure 2.6 and Figure 2.7. Restriction map, multiple cloning sites and the SP6 and T7 transcription sites for the pGEM -T Easy® vector. Figure 2.6 also shows the location of the ampicillin resistance gene and the LacZ gene necessary for the blue/white colony discrimination.

2.6.3 Isolation of Plasmid DNA

Plasmid DNA was isolated from fresh overnight cultures using a commercially available kit, Miniprep® (Qiagen), according to the manufacturer's protocol. Briefly, 1.5ml of culture was pelleted in a 1.5ml microcentrifuge tube. The supernatant was decanted and a further 1.5ml of culture pelleted. The cell pellet was re-suspended in 250µl buffer P1 until a uniform suspension with no clumps was obtained. Lysis buffer P2 (250µl) was added to this and mixed by gentle inversion and the reaction allowed to proceed for three minutes. The reaction was inactivated by adding 350µl of buffer N3 and mixing well. The suspension was centrifuged in a desktop centrifuge at 13,000 rpm for 5 minutes and the supernatant transferred onto a Miniprep® spin column. The silica bound plasmid DNA was washed with 350µl RPE buffer and the DNA was eluted in 50µl elution buffer solution. Eluted plasmid DNA was stored at minus 20°C until use.

Successful ligation and transformation of the Plasmid with the correct DNA fragment was confirmed by restriction digest using the EcoRI or NotI endonuclease to check for the DNA insert fragment size. Agarose gel separation and visualization was used

to confirm expected restriction digest products. The extracted plasmid DNA was then submitted for sequencing. Sequencing for TLR1-10 was done by Ian Bennet, MPU, Royal (Dick) School of Veterinary Studies (RDSVS), Edinburgh. For MyD88 and the other PRRs, the sequencing reaction was done 'in house' followed by submission to the Oxford zoology department for automated sequencing.

Resultant sequences were then subjected to a BLAST (http://www.ncbi.nlm.nih.gov/BLAST/index.shtml) database similarity search program (Altschul *et al.*, 1990) in order to check for closest available sequence matches found in the database. These sequences were used for developing real-time quantitative PCR assays and also used to generate nucleotide and amino acid identity alignments.

2.7 Quantitative Real Time Assay Development

Two-step, quantitative real-time RT-PCR (qPCR) was carried out using a Rotor-Gene™ 3000 Sequence Detection System employing the SYBR Green I technology. Two-step qPCR was preferred because it is technically more practical and reliable and the same batch of cDNA can be used to assess the relative expression of several genes. Each real-time PCR reaction was done in a total reaction volume of 20µl.

2.7.1 Internal PCR primers for real time PCR

Primers were designed using the sequences obtained from the cloned DNA fragments and the web-based Primer 3 design program. The primers for the real time PCRs were 20/21 mers with resultant amplicons between 100 to 170 base pairs. All selected primer sequences were then checked for possible cross-hybridization by subjecting them to a BLAST database similarity search program (Altschul *et al.*, 1990). Prior to being used for the real time PCR, the primers were subjected to normal RT-PCR using full-length cDNA and initial source PCR product as template. Each primer pair resulted in a single distinct band of the expected size upon amplification. Plasmids carrying the outer PRR sequenced fragment were used for

standard curve generation for each PRR gene of interest. All real time PCR primers used in the study are shown in Table 2.5.

Table 2.5 Quantitative RT-PCR Primers Used

Primer Name and sequence	Annealing temp	Product size	Ovine Accession#
Toll-like receptor 1			
TLR1 For 5`- GCACCACAGTGAGTCTGGAA TLR1 Rev 5`- CTGGAGGATCGTGAAGAAGG	62.5	164 bp	AM231298
Toll-like receptor 2			
TLR2 For 5`- GCACTTCAACCCTCCCTTTTA TLR2 Rev 5`- TCTCCGAAAGCACAAAGATG	62.5	125 bp	AM183218
*Toll-like receptor 3****			
TLR3 For 5`- AAAGGGACTTTGAGGCAGGT TLR3 Rev 5`- TAGCTTGCTGAACTGCATGG	62.5	150 bp	AM231299
Toll-like receptor 4			
TLR4 For 5`- TGCTGGCTGCAAAAAGTATG TLR4 Rev 5`- CCCTGTAGTGAAGGCAGAGC	62.5	149 bp	AM231300
Toll-like receptor 5			
TLR5 For 5`-CCTCGAAGCCTTCAGTTACG TLR5 Rev 5`-ACCCTCTGATGGACTGATGC	62.5	121 bp	AM231301
Toll-like receptor 6			
TLR6 For 5`- GCTTTCTTTGGAAACACTGGA TLR6 Rev5`- GAGTCAGTGAGGGCATTTGA	62.5	123 bp	AM231302
Toll-like receptor 7			
TLR7 For 5`- TGGAAAATCTTTCCCAGAGC TLR7 Rev 5`- TCCACTTTTTCATCCATGAGC	62.5	133 bp	AM231303
Toll-like receptor 8			
TLR8 For 5`- CACCAAAGACGCTTCTGTCA TLR8 Rev 5`-GCATGAGGTTGTCGATGATG	62.5	132 bp	AM231304
Toll-like receptor 9			
TLR9 For 5`- AGATGTTTACCCGCCTCTCC TLR9 Rev 5`- CCATGGTACAGGTCCAGCTT	62.5	144 bp	AM231305

N.B. Table of primers used for qPCR continued on the following page.

Table 2.5 Quantitative RT-PCR Primers Used (continued)

Primer Name and sequence	Annealing temp	Product size	Ovine Accession#
Toll-like receptor 10			
TLR10 For 5'- CAAGGATAGGCGTAAATGTGG TLR10 Rev 5'- TGATCAGAGAGACTGCAGAACC	62.5	146 bp	AM231306
Beta actin			
β-actin For 5'- CTGGCACCACACCTTCTACA β-actin Rev5'- CCACATACATGGCAGGAGTG	62.5	149 bp	U39357
MyD88			
MyD88 For 5'- CCCTCGGATAAATGACATGG MyD88 Rev 5'- CAGACACGCACAACTTCAGC	62.5	170 bp	AM117196
CD14			
CD14 For 5'- ATGTGTCTGGCCCAGTGC CD14 Rev 5'- CTCCCTGCTTAGCTTGTTGC	62.5	124 bp	AM117197
*CARD15 (NOD2)****			
CARD15 For 5' - CCACGTCCAGGATGAAGG CARD15 Rev 5'- GAGCCAGACTTCCAGAATGG	62.5	159 bp	AM117125
*Dectin-1****			
Dectin-1 For 5' - CCTACCAAGGCTCTCACGAC Dectin-1 Rev 5' - CCCAGTTGAAAGCATCGTCT	62.5	139 bp	AM167930
*Dectin-2*** α- isoform*			
Dectin-2 For 5' - GAAGGGACAAGGGTGACAGA Dectin-2 Rev 5' - TTGATCACCACCAAGTGAGC	62.5	154 bp	AM167931
*SDHA***			
SDHA For 5' - ACCTGATGCTTTGTGCTCTGC SDHA Rev 5' - CCTGGATGGGCTTGGAGTAA	62.5	126 bp	NM174178

*** These primers span an intron(s) based on BLAST results of the bovine genome

** These primers were kindly provided by other members of the Lab group.

2.7.2 Standard curve generation for PRR assays

2.7.2.1 Linearization of the plasmid DNA

Stocks of PRR plasmid clone standards were generated by linearizing plasmid DNA constructed from outer primers' amplicons (Section 2.6) with the enzyme NdeI (Except for β-actin where XmnI was used). Linearized stock plasmid was used for producing templates for qPCR standards by ten-fold serial dilution and qPCR amplification. Each 1μg of plasmid DNA was cut with 2 units of NdeI incubated for two hours at 37ºC. The PGEM T-Easy vector has one restriction site for NdeI (Figure 2.6 and Figure 2.7) and all PRR amplicons contained no restriction site for the NdeI. Linearization was verified by agarose gel electrophoresis of 5μl of plasmid DNA to show a single band of the expected size (amplicon size plus 3.015Kb vector). Bulk linearized plasmids were purified using a commercially available kit QIAquick® (Qiagen) and stored at minus 20 ºC until use.

2.7.2.2 Quantification of Plasmid DNA - Calculation of Copy Numbers

Plasmid DNA concentration was measured by spectrophotometric determination of nucleic acid content. Molecular weights calculated were calculated based on the total size of the vector plus the PRR amplicon insert. These values were changed into the copy numbers of plasmid based on Avogadro's number (1 mol=6.022×10^{23} molecules) and a consensus between two related formulae;

A formulae according to (Whelan *et al.*, 2003)

Weight in daltons (g/mol)
= (bp size of ds product) (330 Da x 2nt/bp)

Hence: (g/mol)/Avogardro's number
= g/molecule = copy number
(where: bp= base pairs, ds= double-stranded, nt=nucleotides)

and,

according to (Giulietti *et al.*, 2001) and (Overbergh *et al.*, 2003) where;

1μg of 1000bp DNA = 9.1×10^{11} molecules

2.7.2.3 Standard Curve Generation

In order to determine the detection limit of the SYBR green assay and to establish a standard curve used for quantification, serial 10-fold dilutions of Plasmid DNA templates from each PRR (containing the partial sequence of the PRR gene of interest) at a final concentration from 4 x 10^9 copies to 4 x 10^0 per 20µl of PCR reaction were analyzed using the real-time PCR. The standard curves demonstrated a linear relationship across a range of at least 8 logs of plasmid DNA concentrations. The correlation coefficient was between 0.9 to 0.99 with a slope value of the standard curves in the range of -3.33 +/- 0.3 and the PCR efficiency calculated from slope of > 0.9 (Figure 2.8).

2.7.2.4 Quantitative real-time PCR SYBR Green master mix

Quantitative real-time PCRs were carried out in a final volume of 20µl containing 2µl of template cDNA. A master mix containing the gene specific primer pair, dNTPS, Magnesium chloride, nuclease free water, SYBR green and Fastart® Taq was made up as outlined in Table 2.6 below. The master mix was made up on ice and kept on ice until dispensing into the Rotor-Gene™ tubes. Rotor-Gene™ tubes were placed into a pre-chilled aluminium Rotor-Gene block rack and 18µl of master mix placed into each tube. This was then followed by the addition of 2µl of template (PRR plasmid standards dilution series, sample cDNA, nuclease free water as a negative control) in each respective tube and thermocycling on the Rotor-Gene™ 3000.

Table 2.6 SYBR green Quantitative real time PCR 'Master mix'

Reagent	Each	Mix (Z+1) reactions
Taq	0.15µl	(Z + 1) x 0.15µl
Primers (**pmol) Forward	0.2µl	(Z + 1) x 0.2µl
Primers (**pmol) Reverse	0.2µl	(Z + 1) x 0.2µl
dNTPs	0.4µl	(Z + 1) x 0.4µl
$MgCl_2$	2.8µl	(Z + 1) x 2.8µl
Buffer	2.0µl	(Z + 1) x 2.0µl
Nuclease free water (up to 50µl)	11.55µl	(Z + 1) x 11.55µl
SYBR Green	0.7µl	(Z + 1) x 0.7µl
Template (cDNA or Nuclease free water)	2.0µl	
Total vol.	**20µl**	**(Z + 1) x 18µl**

** Optimized working concentration of the primers – See appendix VIII. (Where Z is the total number of PCR reactions required).

All assays were optimized to an identical annealing temperature of 62.5°C and forty cycles. Cycling conditions were; 94°C for ten minutes to achieve activation of the Taq, followed by forty cycles of 62.5°C 20s, 72°C 20s and fluorescence acquisition, followed by 94°C 20s. Optimal performance was achieved by selecting the primer concentrations, and magnesium concentrations, that resulted both in a lower threshold cycle value (Ct), for a fixed amount of target template, acceptable amplification efficiency and minimum primer-dimer formation.

Copy numbers were determined from the Ct values of each sample in comparison to the copy number values assigned from the plasmid DNA standard. This was done by the Rotor-Gene analysis software. Melt analysis was performed after each qPCR run to assess the specificity of amplification. The specificity of each qPCR assay was further verified by carrying out ethidium bromide gel electrophoresis of the amplification products. qPCRs with copy numbers less than 1000/qPCR reaction were run twice.

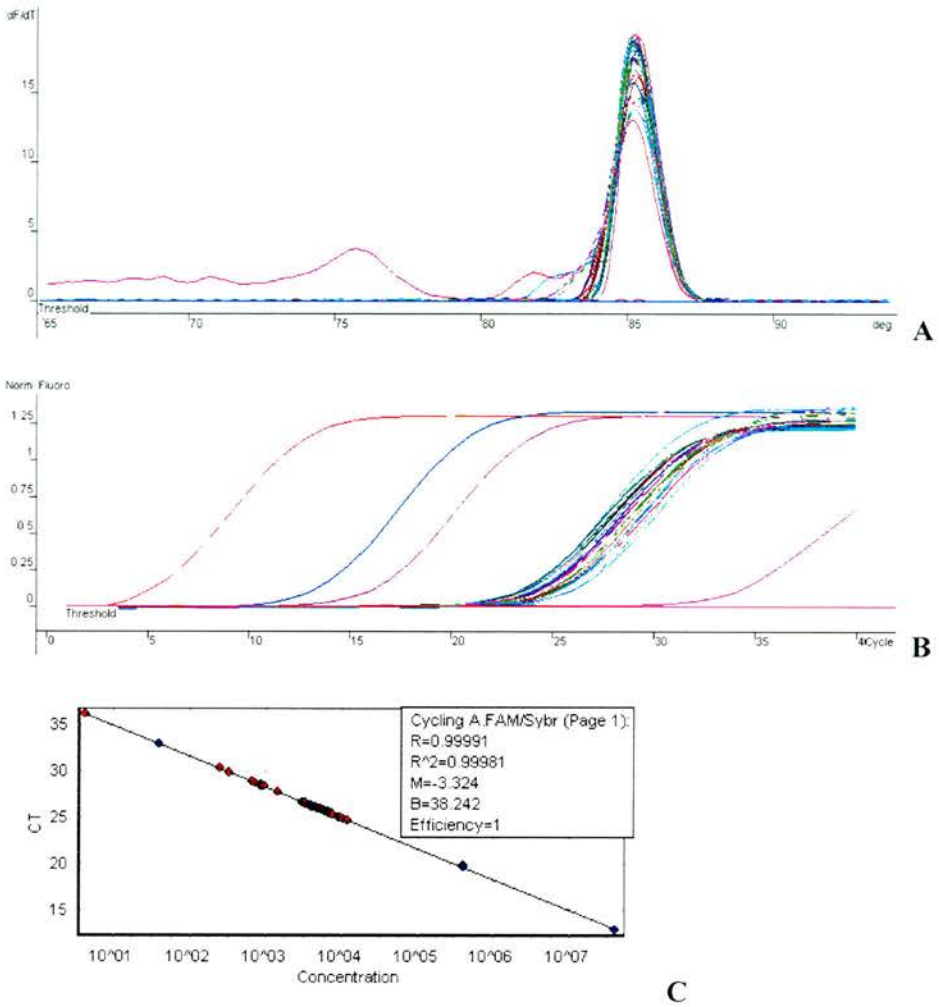


Figure 2.8 Rotor-Gene® quantitative SYBR green Real time PCR melt curves

Examples of quantitative SYBR green dye real time PCR melt curves form an experimental run. A) Showing a single peak on the melt curve with very minimal primer-dimer from the no template negative control. B) qPCR amplification plot of cycle number versus fluorescence intensity C) Standard curve depicted as a plot of log concentration versus CT value. The curve has an ideal slope of 3.324, and efficiency of 1 and a CT intercept of >35.

2.7.2.5 Determination of Intra - assay variation

To determine the accuracy and reproducibility of each qPCR assay, the intra-assay accuracy was determined using triplicates of ten-fold diluted cDNA within one run. The variability was determined by making three ten-fold dilutions the same cDNA and running these cDNAs against the plasmid DNA standards. All comparisons of gene expression in this study were made between samples on the same run and never between runs.

2.7.2.6 Data Analysis - Normalization

To take into account variations in the percentage of messenger RNA present in the starting amount of total RNA, copy numbers from each PRR qPCR run were normalized using β-actin and succinate dehydrogenase (SDHA) housekeeping genes. A gene expression normalization factor taking into account the geometric means of both housekeeping genes was calculated using the geNORM® software (Vandesompele *et al.*, 2002).

2.8 PRR mutation analysis

Forty ileum tissue samples were obtained from Dr. Craig Watkins, (Moredun Institute, Edinburgh) to carry out a pilot search for PRR mutations that may be associated with the various clinical forms of paratuberculosis. The distribution of the samples was 12 paucibacillary, 16 multibacillary and 12 asymptomatic. TLR2 Exon2 and CARD15 exon 11 were chosen based on published literature associating mutations in these genes' exons to the related mycobacterial diseases leprosy (Bochud *et al.*, 2003;Kang and Chae, 2001) and tuberculosis (Ogus *et al.*, 2004) and the granulomatous inflammatory bowel disease (Ogura *et al.*, 2001). These granulomatous conditions in humans have similar immuno-pathological manifestations to ruminant Johne's disease.

Table 2.7 Primers used in the analysis of CARD15 Exon11 and TLR Exon2 for mutations

Primer Name and sequence	Annealing temp (°C)	Product size
CARD15(NOD2) Exon 11		
CARD15 – For 5`- TCATTGGGAATCTCAGACAGG CARD15 – Rev 5`- GAACCAGATTCATCCCATGC	57	378
Ovine TLR2 Exon 2 (Partial)		
DNA1TLR2 – For 5' - TTTCTCATCTCCCAAATCTGC DNA1TLR2 – Rev 5' - AATGGCCTTCTTGTCAATGG	59	1801
DNA2TLR2 – For 5' - TGTGGAGACGTTAACAATACGG DNA2TLR2 – Rev 5'- TCATCAAAGAGACGGAAATGG	59	1256
DNA4TLR2 – For 5'- TGCCTGAAACTTGTCAGTGG		Unidir'
DNA4TLR2_753 5'- ACGCCTTTGTGTCCTACAGC		Unidir'
Unidir' – Unidirectional sequencing primer only		

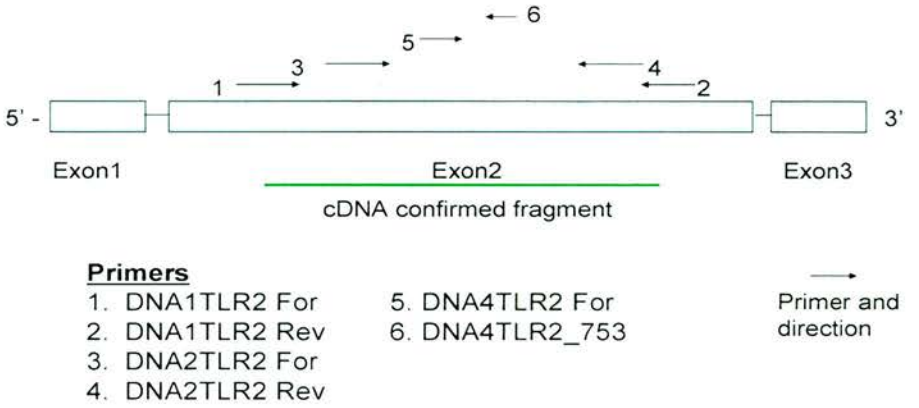


Figure 2.9 Approach to sequencing Ovine TLR2 Exon2 (Accession AM117123).

Each blue box represents an exon as described for bovine TLR2. The arrows associated with a number represent primers that are named. The sequences of these primers are outlined in Table 2.7. The green line represents the fragment of the exon DNA that has been confirmed by PCR amplification from mRNA (cDNA) and sequencing (Accession AM183218).

2.8.1 PCR analysis of genomic DNA

PCR amplification of the forty gDNA samples were performed with each of the primers pairs; CARD15 For/Rev, DNA1TLR2 For/Rev and DNA2TLR For/Rev as outlined previously (Section 2.4.3) with the annealing temperatures shown in Table 2.7 and prolonged extension times of 2 minutes. Primers were designed to result in mutually overlapping fragments (see Figure 2.9) within the TLR2 gene primer set to facilitate sequence assembly. PCR amplification was verified by ethidium bromide gel electrophoresis to ensure the presence of a single distinct amplicon band. The resulting amplicons were purified using the QIAquick® (Qiagen) kit as outlined before (Section 2.5.2) and used as templates for sequencing.

2.8.2 Amplicon Sequencing

PCR amplicons were genotyped by direct sequencing using BigDye® Terminator Cycle Sequencing Kit using both the forward primer and reverse primer. Each sequencing reaction was set up in a thin-walled PCR tube as outlined in Table 2.8.

Table 2.8 Set up of a typical sequencing reaction

Reagent	**Volume (µl)**
Big Dye	0.5
Buffer	1.75
1/100 diluted primer[8]	3.2
Template cDNA (10-40ηg)	1.0
Water	3.55
Total	**10**

The reaction was then run in the thermocycler with thirty cycles of the following cycle: 96°C, 10 sec; 50°C, 5 sec; 60°C, 2 min. After thermocycling, the reaction underwent precipitation and clean up prior to submission for sequencing. Briefly, the reaction was transferred into a clean 0.5ml microcentrifuge tube containing 15µl nuclease free water. To this 50µl 100% ethanol and 2µl 3M sodium acetate (pH 5.2) were added and mixed gently. The mix was incubated at room temperature for 45min followed by precipitation through centrifugation at 13,000 rpm for 30 min in a desktop centrifuge.

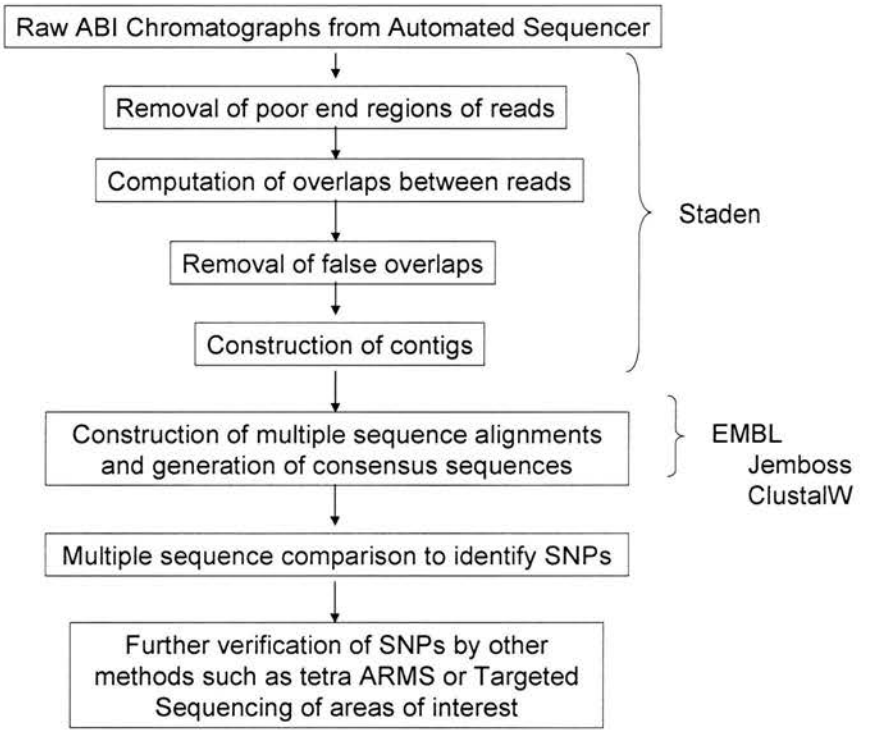
The supernatant was removed carefully, 300µl 70% (v/v) ethanol added and the pellet re-suspended by vortexing. Precipitation was then achieved by spinning at 13,000 rpm for 10 min. The supernatant was removed carefully and the pellet dried by vacuum centrifugation. Sequencing reactions were stored at -20°C until they were sent off to the University of Oxford, Department of Zoology for sequencing.

[8] Amplicon specific primer (reverse or forward) or SP6 or T7 for P-GEM T Easy inserted amplicons

2.8.3 Sequencing data processing

Raw data coming directly from automated sequencing machine often have base-calling errors and require processing for subsequent genomic data analysis. DNA sequence chromatograms were analysed using the Staden® sequence assembly package to distinguish poor quality data from good and to minimize base calling errors. The package was utilised to distinguish regions with trace artifacts due to sequencing chemistry and background noise.

Contiguous regions (contigs) were assembled for each genomic DNA sample and the resultant sequences aligned for comparison and determining possible SNPs. Each of the SNPs determined from sequence comparison were confirmed by manual inspection of the chromatographs. Below is the flow chart representing the various processing stages:



2.8.4 Tetra primer Amplification Refractory Mutation System (tetra-ARMS).

For those amino acid-changing SNPs of interest determined by direct sequencing further verification was sought using another methodology. A multiplex PCR assay was designed to simultaneously amplify the two different alleles in a single PCR reaction. Tetra-ARMS was set up as described by Ye and colleagues (Ye *et al.*, 2001) and primers were designed using the web-based tetra-ARMS primer design software located at http://cedar.genetics.soton.ac.uk/public_html/primer1.html. The primers used are outlined in Table 2.9 and the process is schematically presented in Figure 2.10.

SNP	Primer	Allele	Product size
$A^{182}C$	5'- GTTGTATGTGCCAAAGAGTTTAAAGT	Outer Forward	194 bp
	5'- TAACTGATGTATTAATTTCACTGATGGA	Outer Reverse	
	5'-GCAAATTAGTATCTCTCAGTTCTAAATGAT	A allele	143 bp
	5'- ATTCTTATAGATATTGTAAGTTCCTTGGC	C allele	110 bp
$T^{1516}C$	5'- TGGTACATGAAGATGATGTGGGCCT	Outer Forward	351 bp
	5'- GCAGCATCGTTGTTCTCATCAAAGAGA	Outer Reverse	
	5'- GGAGCTGGAGCACTTCAACCCTCACT	T allele	214 bp
	5'- AAGTCTCGCTTATGAAGACACAGCTTCAG	C allele	192 bp

Table 2.9 Primers used for tetra-ARMS for TLR2 exon 2 SNPs and expected allele specific products

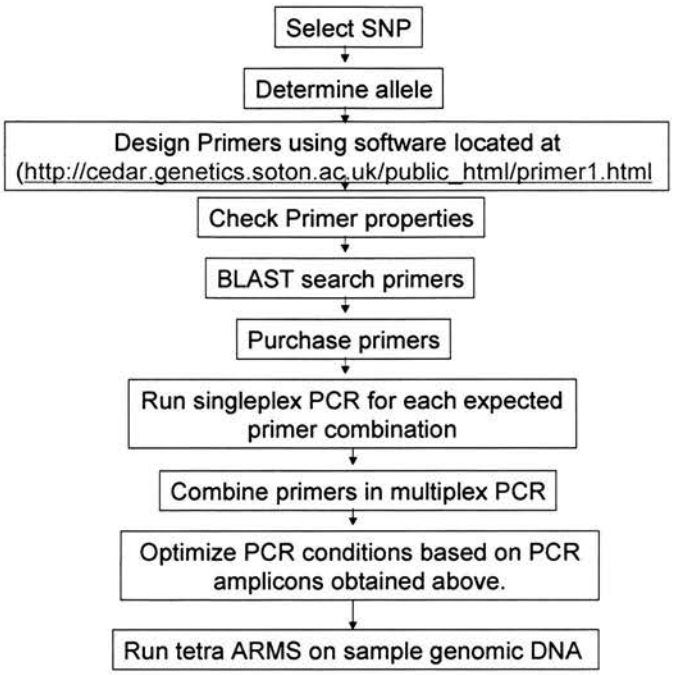


Figure 2.10 Schematic overview of the tetra primer ARMS experimental process

2.9 Data Analysis and Statistical Analyses

Statistical analysis and data graphing was done using MINITAB® Release 14 software for Windows®.

Where data were not normally distributed, Log transformation was carried out to correct the skewed data. Individual values of copy numbers per gene were Log_{10} transformed for data presentation and statistical analysis. Group means for the three clinical types of paratuberculosis were compared using the one way analysis of variance (ANOVA). Tukey's multiple comparison test was used for the pair-wise comparison between the clinical types. Significant differences were defined as those with $p < 0.05$.

3 Identification of ovine Pattern Recognition Receptors

3.1 Introduction

The important role played by PRRs in host-pathogen interactions is well known and it is also known that expression levels change during infection and disease (Akira and Hemmi, 2003; Berrebi *et al.*, 2003). PRR engagement alerts the innate immune system to the presence of 'danger' or microbial infections and eventually culminates in the initiation of adaptive immune responses. Detailed studies of the contribution made by these receptors in disease require accurate quantification of their expression. Few monoclonal antibodies to ovine PRRs exist; therefore, expression of PRRs requires the measurement of PRR messenger RNA (mRNA) transcripts. Although mRNA expression does not always indicate the presence of protein, RNA expression directly correlates to protein production as without mRNA there would be no protein. The aim of this work was to identify ovine PRR homologues to those described in humans and mice and to use the resultant sequences to develop qPCR assays for accurate quantification of ovine PRR gene expression.

Reverse transcription – real time polymerase chain reaction (RT-qPCR) is the most sensitive and accurate technique for mRNA detection, comparison and quantitation currently available. RT-qPCR uses fluorescent reporter molecules that bind to double stranded DNA to monitor the amount of PCR product at the end of each cycle. In comparison to other frequently used research techniques for quantifying mRNA, such as Northern blot analysis, RT-qPCR can be used for the quantification of nominal amounts of mRNA derived from much smaller samples, and with a shorter turn-around time. It is a single homogenous assay that detects fluorescent signal formed proportionally during amplification of a PCR product and obviates the need for laborious post-PCR processing such as gel electrophoresis. There is however, a need for a fully homologous sequence in order to develop reliable, highly reproducible, sensitive qPCR assays.

Since no sheep PRR (of the PRRs studied, other than ovine CD14) sequences were known at the start of this study, the first task embarked on was to obtain these sequences. This was achieved by RT-PCR, designing primers based on the available bovine sequences or where bovine sequences were unavailable, based on consensus

human/mouse sequences. Using these sequences, primers were selected using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). RT-PCR was used for amplification, and assay optimization was carried out for each primer pair, and amplicon size identification carried out using agarose gel electrophoresis. Further verification of the PRR sequences was achieved by restriction digests and via cloning and sequencing. The sequences obtained were finally subjected to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/index.shtml) database comparison. The resulting sequences were used to design primers to be used for the development of quantitative real time RT-PCR assays for PRRs. These assays formed the basis for studying the mRNA expression of PRRs in normal tissues, a Th1/Th2 polarizing disease and in blood & DCs subsets.

3.2 RT-PCR Ovine Pattern Recognition Receptors

As a first step towards determining the gene expression of the different PRRs, qualitative PCR assays were developed for the PRRs using primers designed in Section 2.4.2 and shown in Table 2.2. The results show ethidium bromide stained agarose gels showing separations of PCR product after qualitative RT-PCR using PBMC, spleen and lung derived RNA, with respective PRR primers sets. Amplicon size is estimated by comparison with a 100bp and/or 1,000 bp (1kb) DNA ladder. The expected product size as calculated from bovine and human/mouse consensus sequences are tabulated in Table 3.1.

Table 3.1 Expected PRR amplicon sizes based on bovine and human/mouse consensus sequences

PRR	Expected amplicon		PRR	Expected amplicon
Toll-like Receptors and adaptor molecule MyD88				
TLR 1	375 bp		TLR 6	421 bp
TLR 2	953 bp		TLR 7	752 bp
TLR 3	403 bp		TLR 8	358 bp
TLR 4	945 bp		TLR 9	798 bp
TLR 5	404 bp		TLR 10	648 bp
MyD88	542 bp			
Other PRRs				
Dectin-1	455 bp		Dectin-2	658 bp
CD14	695 bp		CARD15	621 bp

3.2.1 PRRs RT-PCR Agarose Gel Electrophoresis

PRR PCR amplicons were visualized using ethidium bromide agarose gel electrophoresis to ensure the amplification of a specific, single product of the expected size. The gels were visualized on a UV light trans-illuminator and the fluorescent ethidium bromide stained DNA pattern captured with a camera.

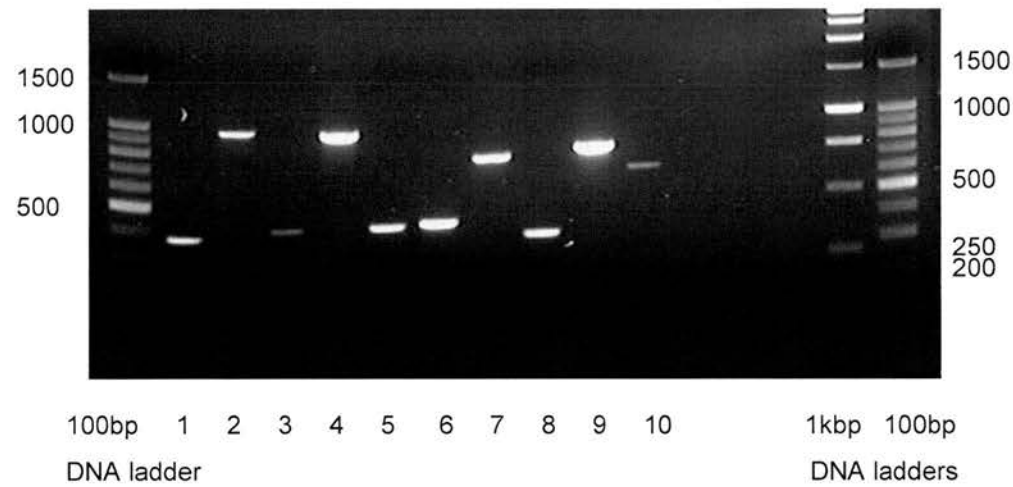


Figure 3.1. 1.5% Ethidium bromide Agarose gel showing all TLR PCR amplicons

Ethidium bromide agarose gel of TLR amplification products. The first and last two lanes represent the molecular size markers (100bp, 1kilo base pair and 100bp respectively). Lanes labelled 1-10 represent each TLR1 - TLR10 respectively. The DNA fragments for all the PCR products are of the expected sizes as estimated from the bovine TLR sequences (Table 3.1).

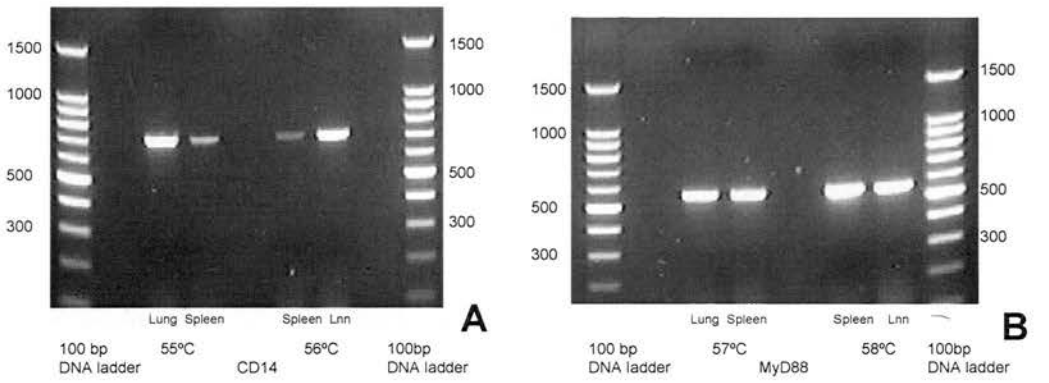


Figure 3.2 Agarose gel of ovine CD14 and MyD88

Ethidium bromide agarose gels showing the PCR amplicons of CD14 (A) and MyD88 (B) on a temperature gradient. **A)** Agarose gel showing the *circa* 700bp PCR amplicon of CD14 at 55°C and 56°C with lung and spleen derived cDNA. **B)** Agarose gel showing the *circa* 550bp PCR amplicon of MyD88 at 57°C and 58°C with lung and spleen derived cDNA.

Abbreviation: Lnn, lymph node.

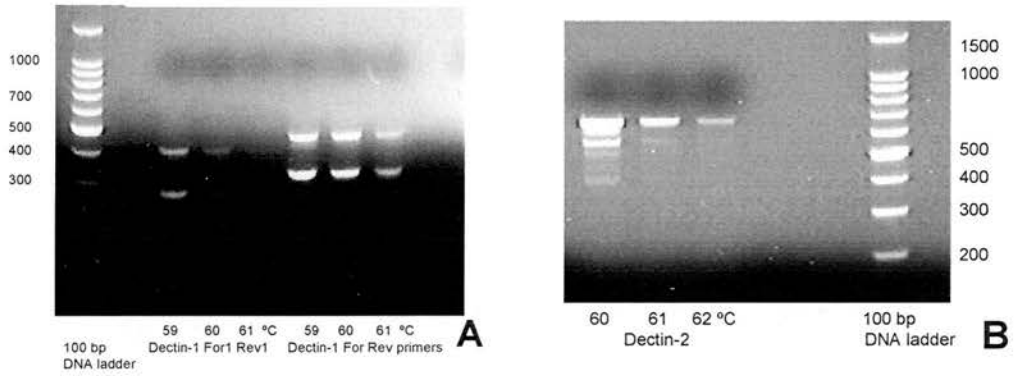


Figure 3.3 Agarose gel of ovine dectin-1 and dectin-2 α and β-isoforms

Ethidium bromide agarose gels showing the PCR amplicons of dectin-1 (*A*) and dectin-2 (*B*) on a temperature gradient. **A)** Agarose gel of dectin-1 isoforms with two different primer pairs and on a temperature gradient. **B)** Agarose gel of dectin-2 showing possible isoforms.

Figure 3.1 shows distinct bands representing ovine TLR1-10 amplicons. All the amplicons are of the expected size as calculated from the source sequences and shown in Table 3.1. The TLR 7 amplicon is of the expected size of *circa* 752 base pairs as compared to the DNA ladders. The gel also shows distinct bands representing the amplicons of TLR2 and 5 which are of the expected size of *circa* 953 and 404 base pairs respectively. The amplicons of TLR1, TLR 6 and TLR8 are *circa* 375, 421 and 358 base pairs respectively which are within the approximate expected product sizes expected.

Figure 3.2A shows distinct bands that represent CD14 amplicons on two annealing temperatures (55°C and 56°C) and using cDNA derived from lung, spleen and lymph node (lnn). The bands are of the expected size of *circa* 695bp and the bands for the lung and lymph node derived cDNA are stronger than those for the spleen perhaps indicating higher transcript content. Figure 3.2B shows distinct bands that represent MyD88 amplicons at two annealing temperatures (57°C and 58°C) and using cDNA derived from lung and spleen. The bands are of the expected size *circa* 542bp. Figure 3.3A shows the two distinct bands represent the long and short forms of dectin-1. The short form is an equivalent of the bovine splice variant that has 138 nucleotide bases (representing exon3) missing (Accession AY937382) as compared to the long form (Accession AY937383). The primer pair on the right, dectin-1 For Rev, (Figure 3.3A) was used and the long-isoform amplicons were gel-extracted and used for cloning and sequencing. Figure 3.3B shows an agarose gel of dectin-2 amplicon also showing possible isoforms product with annealing temperatures of 60°C and 61°C. Similar to the dectin-1 the possible isoforms have a >100bp difference in size and would be equivalent to the bovine dectin-2 alpha(long) form (Accession DQ176046) and the beta(short) form (Accession DQ176047). One of the obvious disadvantages of gel electrophoresis for analyzing PCR DNA products is that it provides little more than relative benchmarks of the size of the DNA fragments on the gel. The gels do not provide much information regarding the specific characteristics of the sequence amplified. For further verification restriction digests were performed.

3.2.2 Restriction mapping of ovine PRR PCR amplicons

All amplicons so far obtained were of the approximate expected size. However, prior to cloning and sequencing, further confirmation of the identity by restriction mapping were performed. Restriction endonucleases were selected using the source bovine sequence, selecting those enzymes that cut the sequence into dissimilar fragments and fragments greater than 100bp each. Table 3.2 lists the endonucleases that were used for those PRRs that were subjected to restriction mapping, showing the expected fragments (based on source sequences (Table 2.2)) and the product on ovine sequences. Figure 3.4 to Figure 3.7 show the restriction maps for the PCR products of TLR 1, 2, 6, 7,10, dectin-2, CD14 and MyD88.

Table 3.2 Expected DNA fragments from endonuclease digest of PRR amplicons

Gene	Restriction Enzyme	Expected Fragment sizes	Actual Fragment sizes obtained
TLR 1	***HinfI***	156/219	*As expected*
TLR 2	***Pst I***	595/358	*Did not digest*
	Sal I	384/569	*As expected*
TLR 6	***EcoRI***	197/224	*As expected*
	Hinf I	285/136	*As expected*
TLR 7	***EcoRV***	286/466	*As expected*
	Rsa I	512/191/239	NOT *as expected*
TLR10	***PstI***	148/500	*As expected*
	XmnI	310/338	NOT *as expected*
CD14	***PstI***	155/540	*As expected*
	XmnI	319/376	*As expected*
	AvaI	82/157/456	NOT *as expected*
Dectin-2	***DraII***	252/443	*As expected*
	HinfI	200/495	*As expected*
MyD88	***HinfI***	244/298	*As expected*
	XhoI	172/370	*As expected*
	AvaI	166/172/204/	NOT *as expected*

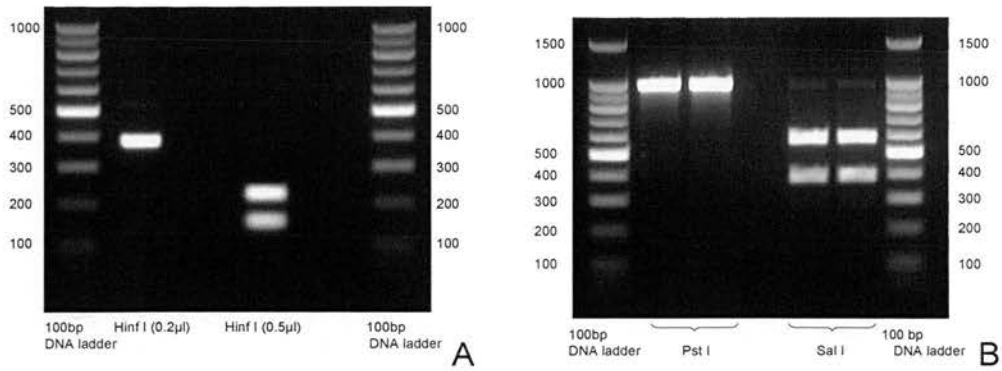


Figure 3.4 Agarose gels of endonuclease digests of ovine TLRs 1 and TLR2

Agarose gel electrophoresis showing restriction maps of ovine TLR 1 and TLR2 amplicons visualized with ethidium bromide staining on 1.5% TAE agarose gels with 100bp DNA ladder **A)** TLR1 endonuclease digestion with HinfI. **B)** TLR2 restriction digestion with Pst I and Sal I.

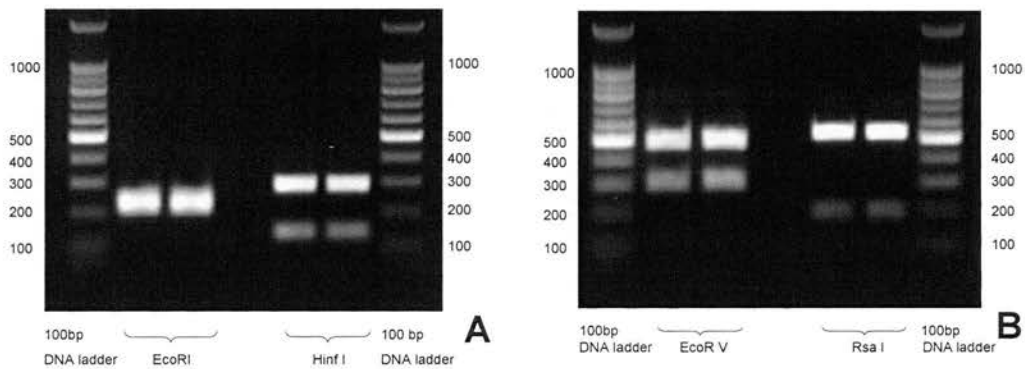


Figure 3.5 Agarose gels of endonuclease digests of ovine TLR6 and TLR7

Agarose gel electrophoresis showing restriction maps of ovine TLR6 and TLR7 amplicons visualized with ethidium bromide staining on 1.5% TAE agarose gels with 100bp DNA ladder. **A)** TLR6 restriction digestion with EcoRI and HinfI endonuclease. **B)** TLR7 restriction digestion with EcoRV and RsaI endonucleases.

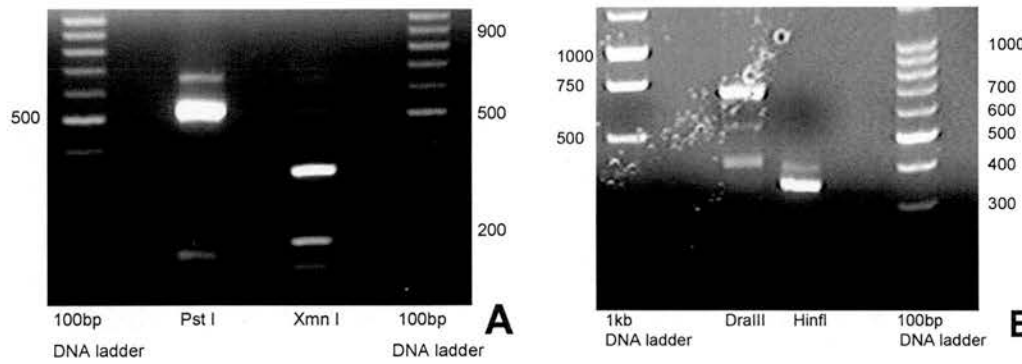


Figure 3.6 Agarose gels of endonuclease digests of TLR10 and dectin-2

Agarose gel electrophoresis showing restriction maps of ovine TLR10 and dectin-2 amplicons visualized with ethidium bromide staining on 1.5% TAE agarose gels. **A)** TLR10 restriction digestion with Pst I and Xmn I visualized with 100bp DNA ladder and **B)** dectin-2 restriction digestion with Dra III and Hinf I visualized with both 1kb and 100bp DNA ladder.

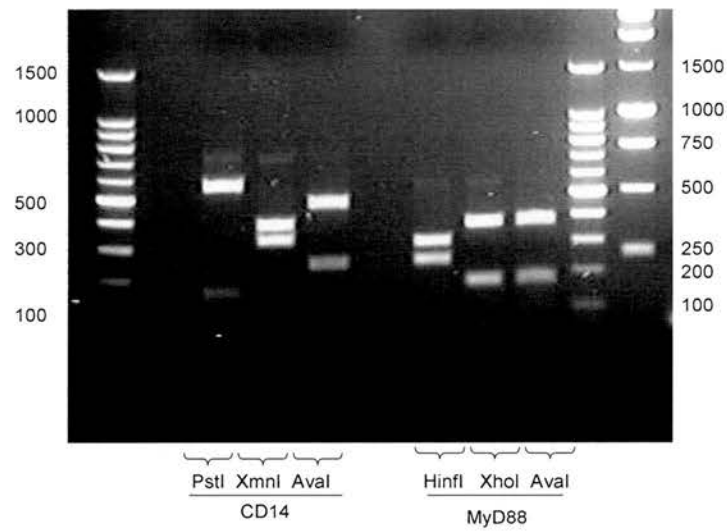


Figure 3.7 Agarose gel of endonuclease digests of CD14 and MyD88

Agarose gel electrophoresis showing restriction maps of CD14 and MyD88 amplicons visualized with ethidium bromide staining on 1.5% TAE agarose gels with 1kb and 100bp DNA ladders. CD14 restriction digestion with Pst I, Xmn I, and Ava I and the MyD88 restriction digestion with Hinf I, Xho I and Ava I.

Following endonuclease digests, the resulting fragment size was determined by agarose gel electrophoresis, it was sometimes not possible to make a distinction between two expected fragments that were within 30bp of each other. This is clearly demonstrated in the TLR6 EcoRI digest (Figure 3.5A) where it was difficult to tell between the 197 and the 224 bp digest product. Some restriction digests that were set up did not cleave the DNA sequences at all and resulted in only the original TLR amplicon being visualized on the gel (Figure 3.4B). Other digests generated maps that were different from what was expected (see Table 3.2) sometimes with more fragments. This was ascribed to differences in the sequences between the bovine (source of the restriction maps) and the ovine sequences (actual DNA being digested). However, the restriction digests provide a useful tool in assisting in verification of DNA sequences. Figure 3.4A shows TLR1 endonuclease digest with 0.2 µl (left) and 0.5 µl (right) HinfI. The left side shows partial digest of the amplicon whilst the right has a complete digestion of the amplicon into the expected product size of 219 and 146/156 bp. TLR2 restriction digest were performed with Pst I and Sal I (Figure 3.4B). The PstI was not able to digest the TLR2 amplicon, even after overnight incubation. Having obtained the ovine TLR2 sequence, it showed that a PstI recognition site is absent in the ovine sequence and that explains why it was not able to digest the amplicon. The Sal I digest shows a faint band of undigested amplicon and distinct bands of the expected digest products sizes of *circa* 384 and 569 bp. Restriction mapping for TLR6 with EcoRI and HinfI endonuclease and demonstrated the expected product sizes of 197/224 and 285/136 respectively. However, the band for the EcoRI does not distinguish between the 197 and 224 bp fragments due to their proximity but the band is wider than the one for the HinfI which has distinct bands. TLR7 Restriction digest (Figure 3.5B) was performed with EcoRV endonuclease and showed a faint band of undigested amplicon and two distinct bands of the expected digest products sizes of *circa* 286 and 466 bp. The endonuclease Rsa I was also used to digest the TLR7 amplicon and it shows two distinct bands one representing the *circa* 512 bp fragment and another wider one representing 191bp digest product. There was however, no fragment representing the 239bp fragment that was expected from Rsa I digestion of bovine TLR7 (Table 3.2).

PstI digest of TLR10 gave the expected products of *circa* 150bp and 500bp (Figure 3.6A). The gel also shows some undigested product of the original amplicon still visible. The XmnI digest of TLR10 was expected to generate two fragments but I got three.

AvaI digest of CD14 and MyD88 gave two restriction digestion products instead of the three that were expected based on the bovine sequence (Table 3.2). The other restriction digestions for the CD14 (with PstI and XmnI) and MyD88 (with HinfI and XhoI) were as expected.

Having putative confirmation of the PRRs' identity using different endonuclease (Table 3.2) it was decided to proceed with cloning and further verification would be achieved post sequencing.

3.2.3 Plasmid Endonuclease Restriction digests

In order to facilitate sequencing, PCR amplicons were purified and cloned into the pGEM-T Easy® vector system (Section 2.6). Following successful ligation and transformation of the PRR inserts into the vector, the structure of the constructs was confirmed by restriction enzyme mapping and DNA sequence analysis. Initial verification of the correct PRR amplicon insertion was done using restriction digest on the circular plasmid using EcoRI and/or NotI endonuclease to excise the insert. After the digestion, ethidium bromide agarose gel electrophoresis was performed to determine the size of the released insert. The endonucleases EcoRI and NotI cut the plasmid DNA in two places thus releasing the insert (Figure 2.6 and Figure 2.7). EcoRI cuts 9bp and 15bp from either side of the vector and similarly NotI cuts 15bp and 21bp on either side and these fragments added to the insert DNA following restriction digestion. This results in restriction maps showing plasmid DNA of *circa* 3.0 kbp and the tagged insert (original size plus 24bp or 36bp (EcoRI, NotI respectively)).

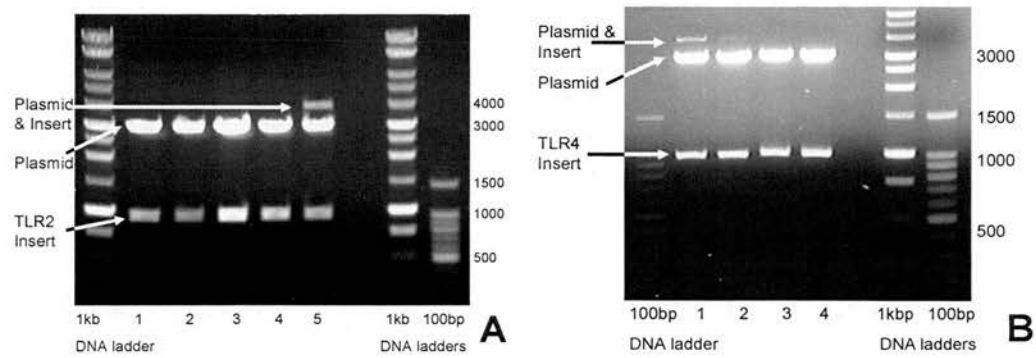


Figure 3.8 Agarose gels plasmid restriction digests TLR2 and TLR4

Agarose gel electrophoresis showing plasmid endonuclease digests of TLR2 and TLR4 visualized with ethidium bromide staining on 1% TAE agarose gels **A)** Ovine TLR2 plasmid digestion with EcoRI **B)** Ovine TLR4 digestion with EcoRI.

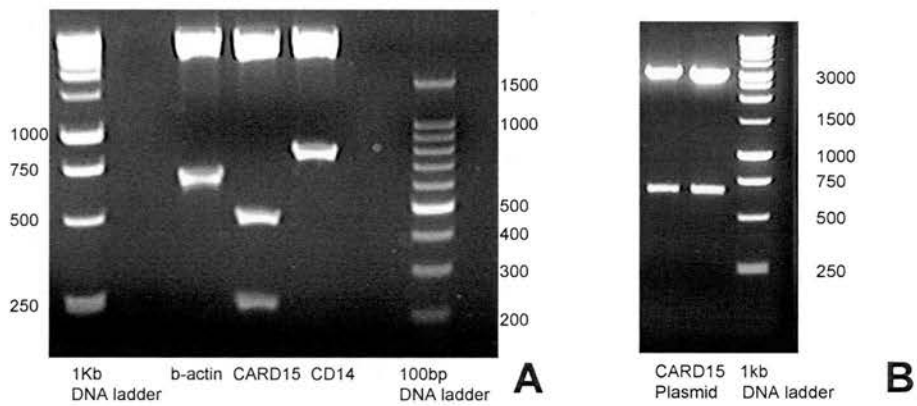


Figure 3.9 Agarose gels plasmid restriction digests β-actin, CARD15 and CD14

Agarose gel electrophoresis showing plasmid endonuclease digestion of β-actin, CARD15 and CD14 visualized with ethidium bromide staining on 1% TAE agarose gels **(A)** Plasmid DNA with β-actin, CARD15 and CD14 amplicon inserts digested with EcoRI. **(B)** CARD15 plasmid DNA repeat digest with NotI.

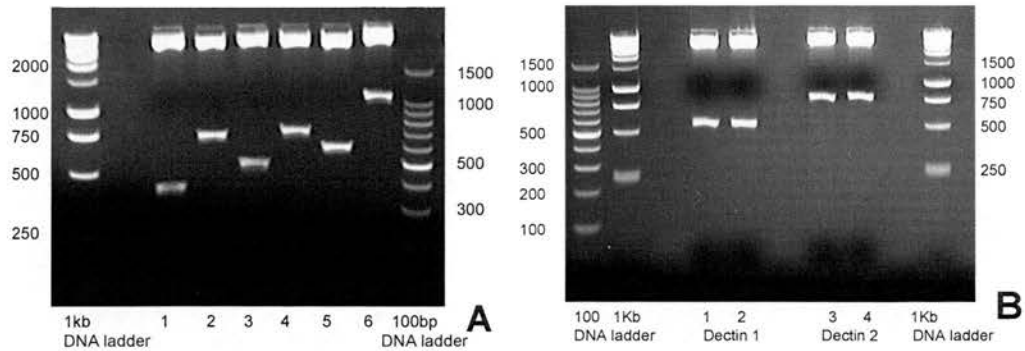


Figure 3.10 Agarose gels Plasmid Restriction digests TLR8, TLR10, CD14, MyD88, PABP, dectin-1 and dectin-2

Agarose gel electrophoresis showing plasmid endonuclease digestion of TLR8, TLR10, CD14, MyD88, PABP, dectin-1 and dectin-2 visualized with ethidium bromide staining on 1% TAE agarose gels **(A)** Lane 1 to 6 representing plasmid DNA digested with EcoRI containing TLR8, TLR10, TLR6, CD14, MyD88 and GAPDH amplicon inserts respectively. **(B)** Plasmid DNA of dectin-1 and dectin-2 digested with EcoRI.

Figure 3.8 to Figure 3.10 show representative restriction maps of ovine PRR and MyD88 plasmid restriction digests with EcoRI and NotI. The maps clearly show the plasmid DNA with the different expected sizes of inserts released, other than for the CARD15. The TLR 2 and TLR4 digestion in Figure 3.8 shows some plasmid DNA that has had only one cut, thus linearizing it but without releasing the insert. This would tend to leave a band with an overall size larger than the pGEM-T Easy vector alone as it would have the amplicon insert still attached. Lane 5 of the TLR2 digest (Figure 3.8A) shows a plasmid DNA fragment of *circa* 4.0kb which would represent plasmid that still has the TLR2 insert attached. The same applied to Figure 3.8B lane 1 and 2.

Figure 3.9A shows the EcoRI restriction maps of β-actin, CARD15 and CD14 plasmid DNA. The released inserts for the β-actin and CD14 were of the expected size but that of CARD15 showed two bands neither being of the correct size. Their cumulative total seemed to be about the expected size of the CARD15 insert. In view of this discrepancy, the CARD15 plasmid DNA digest was repeated with NotI. The NotI digestion of CARD15 revealed the release of the expected size for the CARD15 amplicon and thus was able to confirm that the CARD15 amplicon was inserted (Figure 3.9B). Subsequent sequence analysis demonstrated that the ovine CARD15 has a recognition site for EcoRI and this explains the double bands seen in Figure 3.9A. Figure 3.10 shows the EcoRI digestions release of the inserts of TLR8, TLR10, CD14, MyD88, GAPDH, dectin-1 and dectin-2. The inserts were all of the correct expected size.

Having putative proof that the correct insert was in the vector, plasmid DNA was submitted for sequencing.

3.2.4 Sequences for the cloned PRR amplicons.

The resultant ovine PRR sequences were then subjected to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/index.shtml) database similarity search program (Altschul *et al.*, 1990) in order to check for closest available sequence matches found in the database. The obtained ovine PRR and MyD88 sequences were then used to design primers for the quantitative real time PCR assays for quantifying PRR gene expression. The identity between the ovine nucleotide sequences and the other species' sequences ranged from 57 % to 98% and amino acid identity between 56% to 99%.

3.3 Sequence Analyses of Ovine PRR Sequences

The homologies of ovine PRRs with those of the bovine, human and murine orthologs were compared at the nucleotide and amino acid levels. Sequence comparisons were carried out using the web-based program Emboss: pair wise alignment (http://www.ebi.ac.uk/emboss/align/). For the nucleotide and amino acid sequence identity comparisons, the following criteria were used:

Nucleotide		**Amino Acid**	
Emboss water(local) alignment		Emboss water(local) alignment	
Matrix:	DNAfull	Matrix:	EBlosum62
Gap open:	10	Gap penalty:	10
Gap extend:	10	Extend penalty:	0.5

3.3.1 Sequence comparison analyses for ovine PRRs and MyD88

Multiple sequence comparisons were carried out using the web-based EMBL program ClustalW with default settings and the data was graphically presented and edited using Genedoc® program. Appendix IX shows the graphically presented sequence comparison between the ovine PRR sequences and bovine, human and murine sequences. The human, mouse and bovine sequences were obtained from the public databases at NCBI and compared with the ovine sequences obtained from the present study. Table 3.3 to Table 3.17 summarizes the sequence identities. In the tables the source accession number of the sequences used in the comparison is included and indicated with an asterisk. For the identity values in the table, the figures in italics text represent the nucleotide level sequence identity and those in bold indicate the amino acid sequence identity.

Appendix IX, shows each nucleotide sequence comparison is followed by the respective amino acid identity comparison for each PRR and MyD88. The numbers on the right represent the nucleotide/amino acid positions and on the left of each sequence the species. Identical nucleotide residues for all four sequences are shaded black, three sequences dark grey and two sequences light grey. On the consensus sequence upper case letters further represent agreement of all four sequences and lower case letter represents agreement of three sequences. For the amino acid sequences comparison, Genedoc® graphical presentation is based on similarity/conservation (conserved mode) where black (100%), dark grey (80%) and light grey (60%) amino acid conservation.

Table 3.3 Toll-like receptor one sequence identity summary

Toll-like receptor One	Ovine (AM231298)*	Bovine (AY634628)*	Human (HSU88540)*	Murine (AY009154)*
Ovine	*100 nucl.* AA **100**	*97* **96**	*82* **71**	*75* **69**
Bovine		*100* **100**	*84* **79**	*77* **73**
Human			*100* **100**	*77* **74**
Murine				*100* **100**

Table 3.4 Toll-like receptor two sequence identity summary

Toll-like Receptor Two	Ovine (AM183218)*	Bovine (AF368419)*	Human (NM_003264)*	Murine (BC014693)*
Ovine	*100* **100**	*95* **93**	*83* **75**	*74* **65**
Bovine		*100* **100**	*75* **78**	*69* **67**
Human			*100* **100**	*71* **71**
Murine				*100* **100**

*Accession number

Table 3.5 Toll-like receptor three sequence identities summary

Toll-like Receptor Three	Ovine (AM231299)*	Bovine (AJ812026)*	Human (BC096335)*	Murine (AF355152)*
Ovine	*100* **100**	*98* **98**	*88* **87**	*86* **88**
Bovine		*100* **100**	*83* **82**	*76* **77**
Human			*100* **100**	*80* **80**
Murine				*100* **100**

Table 3.6 Toll-like receptor four sequence comparison summary

Toll-like Receptor Four	Ovine (AM231300)*	Bovine (AY297040)*	Human (HSU88880)*	Murine (AF185285)*
Ovine	*100* **100**	*95* **94**	*80* **74**	*72* **66**
Bovine		*100* **100**	*79* **76**	*73* **65**
Human			*100* **100**	*74* **68**
Murine				*100* **100**

*Accession number

Table 3.7 Toll-like receptor five sequence comparison summary

Toll-like Receptor Five	Ovine (AM231301)*	Bovine (DQ335128)*	Human (BC109118)*	Murine (AF186107)*
Ovine	*100* **100**	*96* **96**	*86* **83**	*81* **72**
Bovine		*100* **100**	*83* **79**	*74* **70**
Human			*100* **100**	*81* **72**
Murine				*100* **100**

Table 3.8 Toll-like receptor six sequence identity comparison summary

Toll-like Receptor Six	Ovine (AM231302)*	Bovine (AY487803)*	Human (AB020807)*	Murine (AF314636)*
Ovine	*100* **100**	*96* **93**	*84* **76**	*76* **72**
Bovine		*100* **100**	*85* **79**	*76* **72**
Human			*100* **100**	*78* **74**
Murine				*100* **100**

*Accession number

Table 3.9 Toll-like receptor seven sequence identity comparison summary

Toll-like Receptor Seven	Ovine (AM231303)*	Bovine (AY487802)*	Human (AF245702)*	Murine (AY035889)*
Ovine	*100* **100**	*98* **98**	*90* **91**	*83* **85**
Bovine		*100* **100**	*87* **86**	*81* **80**
Human			*100* **100**	*83* **81**
Murine				*100* **100**

Table 3.10 Toll-like receptor eight sequence comparison summary

Toll-like Receptor Eight	Ovine (AM231304)*	Bovine (AY642125)*	Human (AF245703)*	Murine (AY035890)*
Ovine	*100* **100**	*96* **98**	*89* **94**	*85* **90**
Bovine		*100* **100**	*79* **73**	*73* **67**
Human			*100* **100**	*75* **71**
Murine				*100* **100**

*Accession number

Table 3.11 Toll-like receptor nine sequence identity comparison summary

Toll-like Receptor Nine	Ovine (AM231305)*	Bovine (AY859726)*	Human (AB045180)*	Murine (AF348140)*
Ovine	*100* **100**	*96* **95**	*83* **77**	*76* **72**
Bovine		*100* **100**	*82* **79**	*77* **74**
Human			*100* **100**	*77* **76**
Murine				*100* **100**

Table 3.12 Toll-like receptor ten sequence identity comparison summary

Toll-like Receptor Ten	Ovine (AM231306)*	Bovine (AY634632)*	Human (AF296673)*	Murine ([1])*
Ovine	*100* **100**	*95* **91**	*83* **75**	
Bovine		*100* **100**	*86* **80**	
Human			*100* **100**	
Murine				

*Accession number

[1] No murine TLR10 sequence has been published

Table 3.13 CARD15 sequence identity comparison summary

CARD15	Ovine (AM117125)*	Bovine (AY518737)*	Human (AF178930)*	Murine (AF520774)*
Ovine	*100* **100**	*96* **97**	*69* **79**	*57* **72**
Bovine		*100* **100**	*77* **81**	*76* **76**
Human			*100* **100**	*69* **79**
Murine				*100* **100**

Table 3.14 MyD88 sequence identity comparison summary

MyD88	Ovine (AM117196)*	Bovine (BC102851)*	Human (BC013589)*	Murine (BC058787)*
Ovine	*100* **100**	*98* **98**	*84* **81**	*78* **71**
Bovine		*100* **100**	*73* **86**	*60* **76**
Human			*100* **100**	*62* **82**
Murine				*100* **100**

*Accession number

Table 3.15 CD14 sequence identity comparison summary

CD14	Ovine (AM117197)*	Bovine (D84509)*	Human (BT007331)*	Murine (BC057889)*
Ovine	*100* **100**	*97* **96**	*79* **74**	*74* **72**
Bovine		*100* **100**	*79* **74**	*60* **62**
Human			*100* **100**	*71* **66**
Murine				*100* **100**

Table 3.16 Dectin-1 sequence identity comparison summary

Dectin-1	Ovine (AM167930)*	Bovine (NM001031852)*	Human (AY026769)*	Murine (AY534909)*
Ovine	*100* **100**	*97* **92**	*81* **72**	*69* **56**
Bovine		*100* **100**	*82* **74**	*71* **59**
Human			*100* **100**	*73* **60**
Murine				*100* **100**

*Accession number

Table 3.17 Dectin-2 sequence identity comparison summary

Dectin-2	Ovine (AM167931)*	Bovine (DQ176046)*	Human (AY365135)*	Murine (AF240357)*
Ovine	*100* **100**	*96* **98**	*82* **77**	*75* **69**
Bovine		*100* **100**	*77* **70**	*65* **66**
Human			*100* **100**	*74* **68**
Murine				*100* **100**

*Accession number

3.4 Results and Discussion

In the last decade, PRRs have generated a lot of interest in immunology research due to their ability to recognize PAMPs and their ability to drive and modulate adaptive immune responses. The existence of PRRs has been established in plants, invertebrates and several vertebrate species. The aim of this study was to determine if selected PRR homologues described in other mammalian species exist in the sheep. Using PCR amplification and cDNA cloning, this study has been able to identify a number of ovine PRRs and MyD88. The data obtained from this study confirms the presence of the ten TLRs hitherto described in humans, mice and cattle and also MyD88, CARD15, CD14, dectin-1 and dectin-2. Bovine and ovine homologues of these PRRs and MyD88 share greater than 95% nucleotide sequence identity and greater than 91% amino acid sequence identity. Sequences identities between the ovine and the murine generally tended to be the lowest in comparison with that of the ovine and other species. The lowest identities with the murine sequences were 57% (nucleotide) and 56% (amino acid) identity.

Sequence comparison allows us to determine the relatedness and evolutionary differences of these genes. PRRs being evolutionary conserved, should have a high percentage of similarity across species boundaries. Recently, Menzies and Ingham (Menzies and Ingham, 2006) have published the identification and partial sequences of the ten ovine TLRs and their sequence comparisons are generally similar with the findings of this study.

As a general observation, the leucine residues locations of the TLRs and CARD15 seem to be more or less conserved within most of the sequences between species.

Ovine dectin-1 and dectin-2 also form α and β-isoforms (long and short forms) as described in cattle (Bonkobara *et al.*, 2006; Willcocks *et al.*, 2006) and humans (Brown and Gordon, 2001; Yokota *et al.*, 2001) as shown by the specific amplification double bands shown RT-PCR (Figure 3.3 A and B). Ovine dectin-2 could possibly also have the γ isoform described in the mouse as evidenced by the multiple bands on the gel. This would need to be verified by cloning and sequencing

the other bands not sequenced for this study. The cloned dectin-1 sequence included the full stalk (α isoform) and partially included the equivalent to the murine CRD domain (part of exon 4, (CRD incorporates exon 4-6)) as predicted by (Adachi *et al.*, 2004) and also the bovine sequence (Willcocks *et al.*, 2006) see Figure 3.11. Similarly, only the long form of the ovine dectin-2 was cloned and sequenced and its amino acid sequence comparison with the bovine long and short forms is shown in Figure 3.12.

Figure 3.11 Bovine dectin-1 α and β-isoforms compared with ovine α-isoform

Figure 3.12 Bovine dectin-2 long and short isoforms compared with ovine long isoform

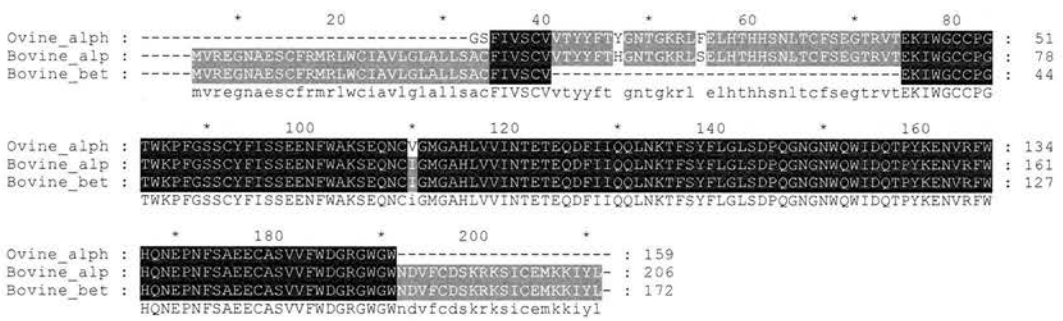


Figure 3.11 and Figure 3.12 Comparison of the amino acid sequences of ovine dectin-1 and dectin-2 with the bovine α and β-isoforms of dectin-1 and dectin-2 respectively.

The ovine PRR sequences that were cloned and sequenced were used to design primers for qPCR assays. Those sequences that included more than one exon (as determined by comparison with the bovine genomic sequence) enabled the design of primers for the qPCR assays that across introns and thus eliminated the possibility of qPCR signal from genomic DNA. The plasmid DNA was used as a template for the generation of qPCR standard curves for 'absolute' copy numbers qPCR quantification.

All the PRR nucleotide sequences that were cloned and sequenced for this study have been deposited in the National Centre for Biotechnology Information (NCBI) public databank, Genbank with the following accession numbers TLR1 (AM231298), TLR2 (AM183218), TLR3 (AM231299) TLR4 (AM231300) TLR5 (AM231301) TLR6 (AM231302) TLR7 (AM231303) TLR8 (AM231304) TLR9 (AM231305) TLR10 (AM231306), MyD88 (AM117196), CD14 (AM117197), CARD15 (AM117125), dectin-1 (AM167930), dectin-2 (AM167931). From the sequences' identity comparison, I therefore conclude that the cloned sequences for ovine TLR1 - TLR10, MyD88, CD14, CARD15, dectin-1 and dectin-2 are the ovine homologues to the human, murine and bovine counterparts. It would be interesting, whenever full sequences of these ovine PRRs become available, to carry out predicted structural analysis and also phylogeny studies.

4 PRRs mRNA expression in normal tissues

4.1 Introduction

The ability of a multi-cellular organism to detect and eliminate pathogens is the most critical task of the immune system. Initial host defence mechanisms are initiated by the innate immune system and this step drives the adaptive immune response. Accurate recognition is essential for tailoring an appropriate immune response to eliminate a pathogen and it is now well-established that the detection of pathogens is done via innate immune receptors called pattern recognition receptors (PRRs). PRRs have evolved to recognize PAMPs and mediate immune responses to microbes in insects and mammals via the induction of protective, pro-inflammatory mechanisms (Janeway and Medzhitov, 2002; Martin *et al.*, 2003). PRRs are also reported to be involved in the maintenance of homeostasis and tolerance development (Rakoff-Nahoum *et al.*, 2004). In order to perform the above functions, PRRs are able to discriminate between, 'non-self', 'self', and 'altered self'. Microbial infection of a mammalian host can have potentially harmful sequela and different tissues and mucosal sites have different immune challenges with some being inhabited by commensal micobes. These differences in level of commensals, and potential microbial exposure would suggest that each tissue would have different innate immune mechanisms to sense and mount an immune response, or induce tolerance.

Having identified selected PRRs and MyD88 in sheep in Chapter 3, this chapter looks at the expression of these different PRRs and MyD88 in selected foetal (skin and spleen) and adult (skin, spleen, kidney, lung, prescapular lymph node, mesenteric lymph node and urinary bladder) sheep tissues. The spleen is a secondary lymphoid organ that has the function of facilitating the interaction between APCs and lymphocytes. The spleen is a major focus for APC/Ag interaction with T cells and also a major centre for B cell congregation. The spleen also has a second and vital function of removing infected, defective and old erythrocytes. Lymph nodes have a similar function and structure to the spleen, but lack red pulp and do not have the function of erythrocyte removal. Lymph nodes and the spleen are extremely dynamic organs and their cellular constitution rapidly changes with immune status. During systemic infection, APC and lymphocyte cell trafficking and cell content in the spleen increases and a similar situation occurs during infection of regions drained

by specific lymph nodes. Mucosal epithelia have innate immune functions by acting as physical barriers and also as the first line of defence by innate immune responses of epithelial cells and sub-epithelial leucocytes. The mucosa of the lung is in constant contact with inspired air that may contain microbes and allergens. Unlike the lung, the mucosa of the urinary bladder is not directly in contact with the externum, though it is in perpetual contact with the urine produced by the kidney and urine contents may be immunostimulatory. Skin and mucosae represent dynamic tissues that must respond effectively and appropriately to constantly changing endogenous and exogenous stimuli. These tissues are able to do this by possessing an array of PRRs of sufficient quantity and quality to recognise and respond to all potential pathogens while maintaining tolerance to commensal organisms.

I postulated that the PRR expression in different tissues would be different to reflect their different immuno-physiological functions and anatomical properties. Innate immune mechanisms initiated by PRRs in different tissues may elucidate each tissues unique immune potential and ability to respond to particular pathogenic challenge. A tissue's PRR expression profile/repertoire would thus represent its ability to accurately recognize pathogens and mount an appropriate immune response. It would indirectly translate into a host tissues' pathogen susceptibility and capacity to eliminate named pathogen classes. The potential risk of pathogen challenge is higher on mucosae thus PRR expression would be expected to be high especially in those mucosae that have a rich commensal flora. Thus, I postulated that the urinary bladder would have lower PRR expression compared to the skin and the lung that have constant contact with the externum, and the skin has a resident commensal flora. I further hypothesized that secondary lymphoid organs like lymph nodes would also be expected to have high PRR expression to reflect the high immunological activity taking place there and the cellular composition of cells known to highly express PRRs such as DCs, macrophages and B cells. The kidney has a very low APC/immune cell infiltrate and I postulated that it would have low PRR expression. The renal epitheliae also does not communicate directly with the externum and would thus have a reduced risk of direct pathogen challenge.

Ligation of most TLRs (other than TLR3) leads to the recruitment of an adaptor molecule, MyD88, and this leads to a signal transduction cascade that, via NF-κB, culminates in immune gene expression. A lack of MyD88 has been associated with severe immunopathologies in mice (Schnare *et al.*, 2001). Thus, taking into account the importance of this adaptor molecule in TLR signalling, the expression of MyD88 in tissues was also analysed. I postulated that tissues with high potential risk of pathogen infection, high PRR expression and high commensal microbiota would also have a high MyD88 expression.

Mammalian neonates are known to have a reduced response to specific antigens and an increased susceptibility to bacterial infections and sepsis. This increased susceptibility is more pronounced in premature babies where it causes significant morbidity and mortality although species differences exist in the level of immune development at birth (Al Salami *et al.*, 1985; Durandy, 2003; Schultz *et al.*, 1973). This has been partly ascribed to neonates having reduced complement factors, poor phagocytic capabilities (Strunk *et al.*, 2004) and an overall underdeveloped innate immune system and naïve adaptive immune system. However, it is evident that other mechanisms might be important in this immune status and are continuingly being investigated. PRRs are a critical aspect of innate immunity and their expression levels could therefore represent a mechanism explaining the underdeveloped immune response of neonates. Literature on mammalian PRRs ontogeny is scanty and disparate findings have been reported on mRNA levels in prenatal tissues compared to neonatal and adult levels with some workers reporting lower neonatal levels and others reporting comparable levels. To ascertain the possible contribution of the foetal innate immune system's pattern recognition receptors to the initiation of antigen specific immune reactivity, a comparative age-related PRR expression was investigated in second trimester spleen (a secondary lymphoid organ) tissue and skin (an organ that is in contact with the external environment in the amnion) in comparison to similar tissue from healthy adult sheep. I hypothesized that PRR expression in the foetus is either absent or very low and expression increases with progressing gestation, with neonatal levels still being significantly lower than adult PRR levels.

4.2 PRRs mRNA expression in normal adult ovine tissues

In order to assess the biological tissue diversity of PRRs, quantitative mRNA expression in seven different normal ovine tissues was analyzed. The tissues were taken from clinically healthy adult sheep and represented secondary lymphoid tissues (spleen, mesenteric and prescapular lymph node), mucosae (lung and urinary bladder), skin and an internal organ (kidney) (Section 2.2.2). The PRR mRNA expression in the ovine tissues was analyzed by SYBR green, reverse transcriptase, real time quantitative PCR technique, a sensitive technique for the detection and quantitative enumeration of mRNA transcripts (Section 2.7). Figure 4.1 shows the PRR expression in the different ovine tissues and Table 4.1 shows the linear means of the tissues compared to spleen PRR expression whilst Table 4.2 contains a more detailed tabulation of the statistical relationships between all the tissues.

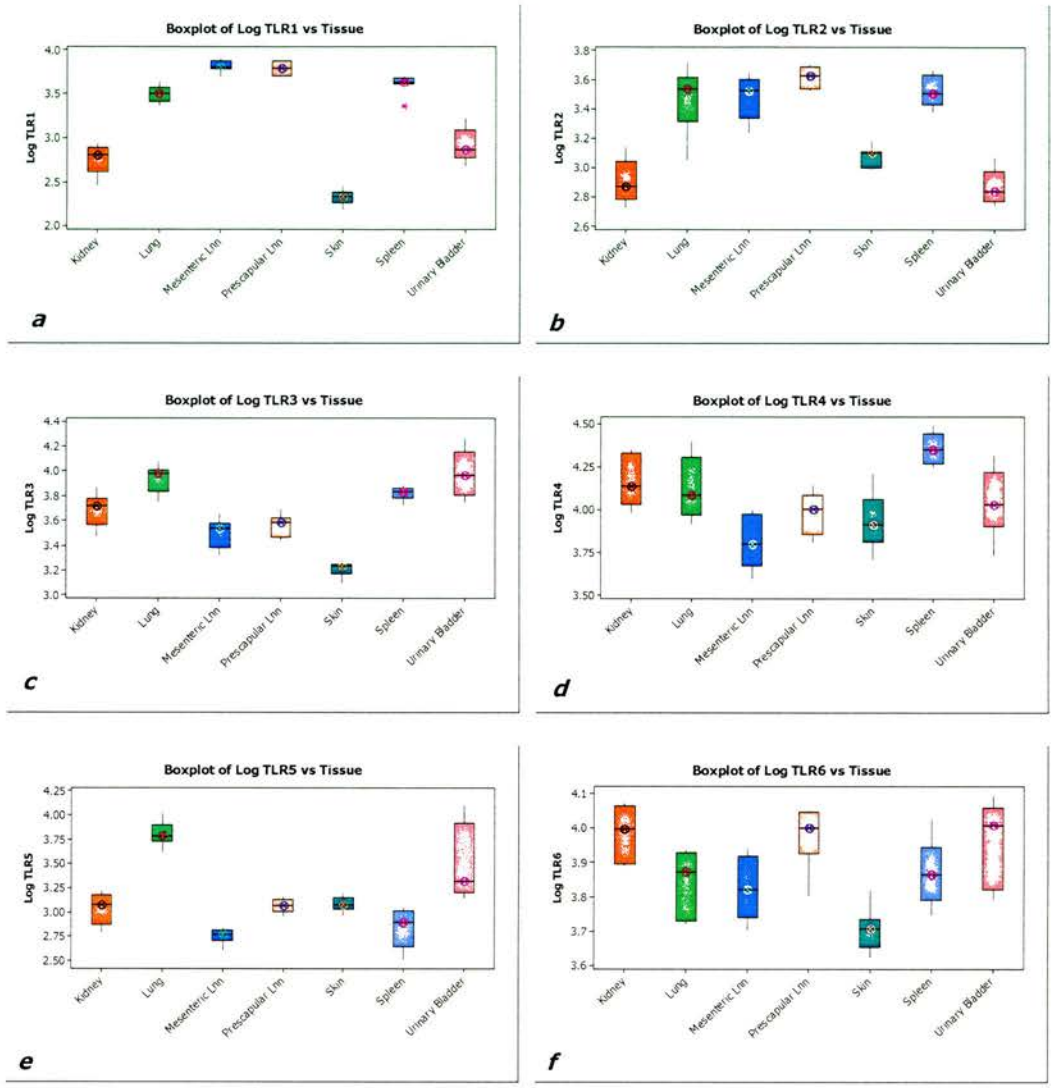


Figure 4.1 PRR mRNA expression in selected normal adult ovine tissues

Graphical representation of the TLR1*(a)*, TLR2*(b)*, TLR3*(c)*, TLR4*(d)*, TLR5*(e)* and TLR6*(f)* mRNA expression in selected normal adult ovine tissues (n=4). Data are expressed as box plots of Log_{10}(normalized copy number/qPCR reaction) of each PRR. The box representing the 25 and 75% percentile, and the circle represents the median and the whiskers representing data range. Where present, the asterix (*) represents an outlier value.

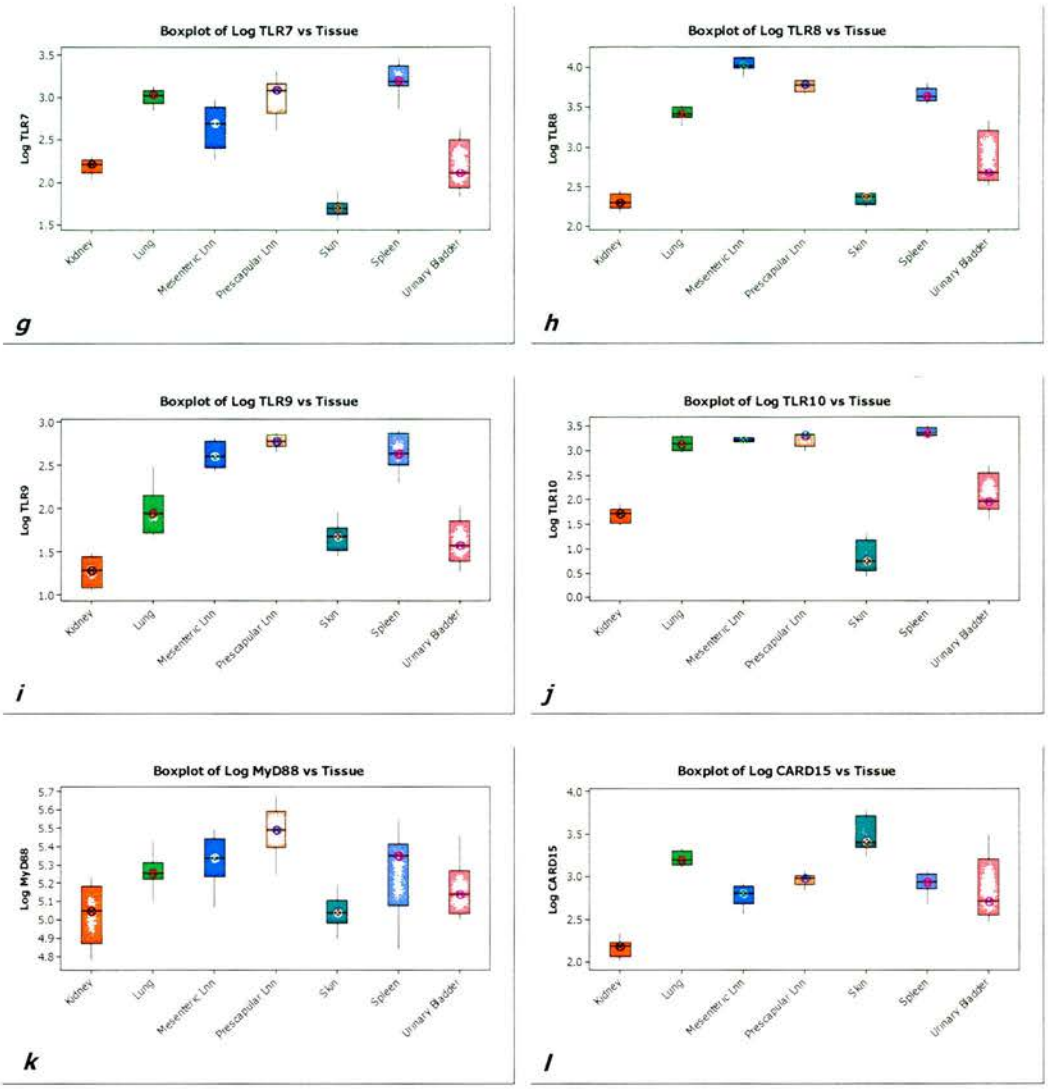


Figure 4.1 PRR mRNA expression in selected normal adult tissues (continued)

Graphical representation of TLR7 ***(g)***, TLR8 ***(h)***, TLR9 ***(i)***, TLR10 ***(j)***, MyD88 ***(k)*** and CARD15 ***(l)*** mRNA expression in selected tissues (n=4). Data are expressed as box plots of Log_{10}(normalized copy number/qPCR reaction) of each PRR. The box representing the 25 and 75% percentile, and the circle represents the median and the whiskers representing data range.

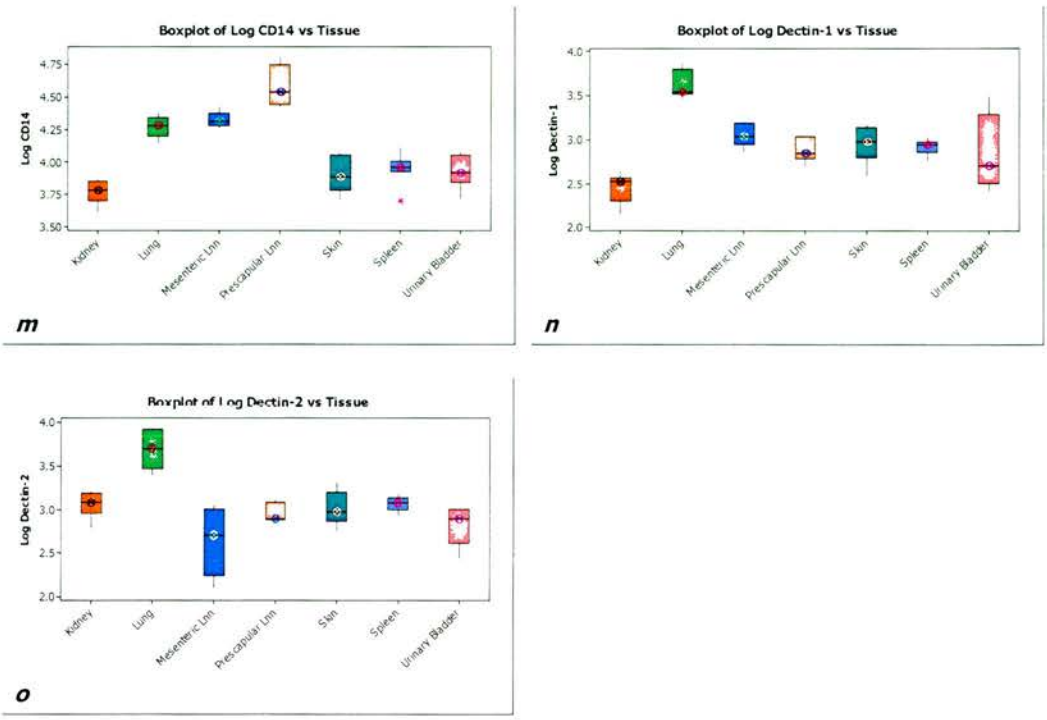


Figure 4.1 PRR mRNA expression in selected normal adult ovine tissues (continued)

Graphical representation of CD14 ***(m)***, dectin-1 ***(n)*** and dectin-2 ***(o)*** mRNA expression in selected tissues (n=4). Data are expressed as box plots of Log_{10}(normalized copy number/qPCR reaction) of each PRR. The box representing the 25 and 75% percentile, and the circle represents the median and the whiskers representing data range. Where present, the asterix (*) represents an outlier value.

TLR1 is the main receptor for microbial lipoproteins and it is also known to form functional heterodimers with TLR2 which widens the receptor's ligand range (Ozinsky *et al.*, 2000; Takeuchi *et al.*, 2001; Takeuchi *et al.*, 2002). Real time quantitative PCR showed that TLR1 was highly expressed in the lymph nodes, lung and spleen. Expression in the kidney and urinary bladder was moderate and only weakly expressed in the skin with the skin having almost twenty fold lower expression than the spleen (Figure 4.1*a*) and (Table 4.1). There was a greater than five-fold difference in expression level between the kidney and the lung and about ten-fold difference between the kidney and the lymph nodes and the difference in mRNA expression was statistically significant between the two groups. Disparity in mRNA expression between the kidney and urinary bladder were not statistically different. Differences in mRNA expression between the skin and the kidney and bladder were also statistically significant. TLR2 recognises the broadest range of ligands that include peptidoglycan from Gram positive bacteria, bacterial lipopeptides, lipoarabinomannans from *Mycobacteria*, and yeast zymosan. TLR2 has a similar tissue expression profile to TLR1 with the lymph nodes, lung and spleen having an approximately five-fold higher expression of TLR2 than the expression of the kidney and urinary bladder. Unlike TLR1, the skin expresses more TLR2 transcripts than the kidney and urinary bladder (Figure 4.1*b*). The skin's mean expression level of TLR2 is statistically higher than that of the kidney and urinary bladder. However, the difference in the mean TLR2 mRNA expression between the kidney and urinary bladder were not statistically different.

The natural ligand for TLR3 is pathogen derived ds RNA and host nucleic acids (Aksoy *et al.*, 2005; Alexopoulou *et al.*, 2001; Kariko *et al.*, 2004). TLR3 was highly expressed in the kidney, spleen, lung and urinary bladder and only moderately expressed in the mesenteric and prescapular lymph nodes and skin (Figure 4.1*c*). There was a four fold difference in expression between with the kidney and the moderately expressed tissues such as the mesenteric and prescapular lymph nodes and skin. The spleen and urinary bladder had about two-fold and three-fold higher expression than the moderate tissues respectively (see also Table 4.1 and Table 4.2).

TLR4 recognises bacterial LPS in conjunction with the accessory molecules, CD14 and MD-2. TLR4 also recognises host derived fibrinogen, Hsp and β-defensins. All tissues examined expressed TLR4 transcripts in considerable quantities with the spleen expressing the highest amounts followed by the kidney and lung (Figure 4.1*d*). The spleen had a greater than two fold higher expression of TLR4 than the lymph nodes, skin and urinary bladder and the difference was statistically significant. The mesenteric lymph node revealed the lowest amount of TLR4 transcripts followed by the skin, prescapular lymph node and urinary bladder, but the differences between these tissues were not statistically significant.

Bacterial flagellin is the described natural ligand for TLR5. The lung expresses the highest amount of TLR5 transcripts, followed by the urinary bladder. TLR5 expression in both these tissues is significantly higher than the other tissues studied but TLR5 expression in the lung is significantly higher than the urinary bladder. Ovine mesenteric lymph nodes and spleen expressed the lowest TLR5 transcripts, being approximately 9 fold lower that the expression in the lung. Prescapular lymph nodes, kidney and skin have expression levels that are approximately two fold higher that that of the mesenteric lymph node and spleen (Figure 4.1*e*).

TLR6 participates in the recognition of diacylated lipopeptides, usually in conjunction with TLR2 (Nakao *et al.*, 2005; Ozinsky *et al.*, 2000) and is expressed in appreciable quantities in all the tissues examined though the skin has a significantly lower expression (Figure 4.1*f*). The difference between the highest expressed tissues (kidney, prescapular lymph node, and urinary bladder) and the skin is approximately two - fold. TLR6 expression levels of the lung, mesenteric lymph nodes and spleen is statistically comparable and about 1.5 fold higher than the skin.

TLR7 and 8 are reported to have viral single stranded nucleic acids as the natural ligands (Heil *et al.*, 2004; Lund *et al.*, 2004). TLR7 transcripts in the skin were just barely perceptible being five-fold less than that in the kidney and urinary bladder (Figure 4.1*g*). The spleen had the highest TLR7 expression, being about 30-fold higher than in the skin, followed by expression in the lung and prescapular lymph nodes that was about 1.5 fold lower than the lung. The mesenteric lymph nodes had

TLR7 expression approximately three fold lower than that of lung but about nine-fold more than the skin and both of these differences were statistically significant (see also Table 4.1 and Table 4.2). Secondary lymphoid tissues and the lung express the highest levels of TLR8 of the tissues examined (Figure 4.1*h*). The mesenteric lymph nodes have the highest TLR8 expression, being about five-fold higher than the lung and about 2.5 fold higher than the spleen and the TLR8 expression in the mesenteric lymph nodes is significantly higher than all the other tissues examined. The kidney and the skin have comparable TLR8 expression but express statistically lower TLR8 transcript levels than all the other tissues, being approximately fifty fold less that that of the mesenteric lymph nodes and three fold less than the urinary bladder.

The kidney had imperceptible expression of TLR9, the receptor for bacterial DNA and host chromatin Tg-complexes. The secondary lymphoid tissues had the highest expression that was more than fifteen-fold greater than that of the kidney. The skin and urinary bladder had comparable, but low levels of TLR9 expression that was about two fold more than the expression in the kidney (Figure 4.1*i*). The lung had six fold more than the expression in the kidney (Figure 4.1*i*) and this difference was statistically significant.

TLR10 has no known natural or synthetic ligand (Chuang and Ulevitch, 2001; Hasan *et al.*, 2005). Similar to TLR9, TLR10 was highly expressed in the secondary lymphoid tissues and also in the lung. The spleen had the highest expression being approximately 1.5 fold higher than lung and mesenteric lymph node and 1.3 fold higher than the prescapular lymph node. TLR10 expression differences between the lung, prescapular lymph node and mesenteric lymph nodes were not statistically significant. The skin had imperceptible expression of TLR10. The urinary bladder and kidney had low expression of TLR10, being about ten fold and fifty fold lower than that in the spleen respectively (Figure 4.1*j*).

MyD88 is an adaptor molecule required for TLR signalling (other than TLR3), as well as interleukin-1 and interleukin-18 signalling (Medzhitov *et al.*, 1998; Wesche *et al.*, 1997). The prescapular lymph node had the highest expression of MyD88, having about 1.5 fold higher mRNA expression than the lung, mesenteric lymph nodes and spleen. The prescapular lymph node had approximately three-fold higher MyD88 transcripts than the skin, kidney and two-fold higher expression than the urinary bladder (Figure 4.1*k*). The MyD88 expression in the skin, kidney and urinary bladder is statistically comparable, but statistically significantly lower than the other tissues.

CARD15 is a PRR that is reported to recognize muramyl dipeptide (MDP) a PGN derivative but is also reported to have a negative regulatory function on TLR2 signalling (Girardin *et al.*, 2003; Watanabe *et al.*, 2004). The skin had the highest expression of CARD15, having transcript levels about twenty fold higher than the lowest expressed tissue, the kidney and a 1.5 fold higher expression than its next ranked tissue, the lung (Figure 4.1*l*). There is no statistical difference in CARD15 expression between the lung and the skin but the skin has a significantly higher CARD15 expression than the other tissues. The lymphoid tissues and the urinary bladder had comparable expression levels that were approximately five to six-fold higher than the kidney.

CD14 is a co-receptor for LPS recognition by TLR4 and is highly expressed on cells of myeloid derivation such as macrophages and monocytes. The prescapular lymph node had the highest expression of CD14 with the kidney having the lowest tissue expression (Figure 4.1*m*). CD14 expression in the prescapular lymph nodes is significantly higher than all the other tissues examined. The kidney had a greater than five-fold lower expression of CD14 than the prescapular lymph node and the lung and mesenteric lymph nodes had an approximately four fold greater expression than the kidney. Skin, spleen and urinary bladder had statistically comparable CD14 expression and an approximately two-fold higher expression than the kidney. The spleen had significantly higher CD14 expression than the kidney, but the skin, urinary bladder and kidney are statistically comparable.

Dectin-1 and dectin-2 are C-type lectins involved in the recognition of sugar moieties. The secondary lymphoid tissues, skin and urinary bladder express similar levels of dectin-1 (Figure 4.1*n*). The lung has a statistically significantly higher expression of dectin-1 than all other tissues. The lung expresses approximately fourteen fold higher dectin-1 than the kidney and about five-fold higher expression of dectin-1 than the lymphoid tissues. The lung similarly expresses, approximately five fold higher expression of dectin-2 than all the other tissues studied and this difference is significant. The mesenteric lymph node had the lowest expression of dectin-2 and the expression was statistically significantly lower than the lung, skin and spleen (Figure 4.1*o*).

Table 4.1 shows data with the all relative fold changes of the mean of the linear absolute mRNA expression of the different PRRs and MyD88, normalized to spleen transcript levels. Table 4.2 shows the detailed statistical relationships between each of the seven different tissues for each PRR and MyD88. Statistical relationships were generated using one-way ANOVA and Tukey's multiple comparison test with statistical significance being a p value <0.05.

Relative PRR Expression	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6
Kidney	0.15	0.25	0.76	0.66	1.54	1.29
Lung	0.77	0.96	1.34	0.61	8.67	0.93
Mesenteric Lnn	1.57	0.94	0.49	0.30	0.75	0.90
Prescapular Lnn	1.51	1.24	0.56	0.43	1.54	1.27
Skin	0.05	0.35	0.25	0.39	1.60	0.67
Spleen	1.00	1.00	1.00	1.00	1.00	1.00
Urinary Bladder	0.21	0.22	1.56	0.50	6.02	1.23

Relative PRR Expression	TLR7	TLR8	TLR9	TLR10	MyD88	CARD15
Kidney	0.09	0.05	0.04	0.02	0.54	0.17
Lung	0.58	0.58	0.23	0.60	0.89	1.92
Mesenteric Lnn	0.28	2.41	0.90	0.69	1.04	0.73
Prescapular Lnn	0.65	1.29	1.24	0.75	1.53	1.08
Skin	0.03	0.05	0.10	0.00	0.54	3.87
Spleen	1.00	1.00	1.00	1.00	1.00	1.00
Urinary Bladder	0.10	0.19	0.10	0.08	0.75	1.16

Relative PRR Expression	CD14	Dectin1	Dectin2
Kidney	0.66	0.36	1.03
Lung	2.08	5.30	4.69
Mesenteric Lnn	2.34	1.36	0.50
Prescapular Lnn	4.42	0.93	0.79
Skin	0.93	1.12	0.97
Spleen	1.00	1.00	1.00
Urinary Bladder	0.97	1.25	0.62

Table 4.1 PRR tissues expression relative to the spleen PRR expression.

Mean PRR expression of different tissues normalized to the spleen mean expression. To facilitate comparison, the spleen mean PRR expression is allocated an arbitrary value of 1 and mean PRR expression of all other tissues are compared to this arbitrary value.

Table 4.2 Statistical relationships between tissue groups.

TLR1

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	-	+	0
Lung		0	+	+	-	0	-
M. lnn			0	0	-	-	-
P. lnn				0	-	0	-
Skin					0	+	+
Spleen						0	-
Ur. Bl.							0

TLR2

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	0	+	0
Lung		0	0	0	-	0	-
M. lnn			0	0	-	0	-
P. lnn				0	-	0	-
Skin					0	+	-
Spleen						0	-
Ur. Bl.							0

TLR3

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	-	0	-	0	+
Lung		0	-	-	-	0	0
M. lnn			0	0	-	+	+
P. lnn				0	-	+	+
Skin					0	+	+
Spleen						0	0
Ur. Bl.							0

Key: 0 : no significant difference - $p>0.05$
+ : column tissue significantly higher than the row tissue - $p\leq 0.05$
- : column tissue significantly lower than the row tissue - $p\leq 0.05$

M. lnn (mesenteric lymph node), P .lnn (prescapular lymph node) Ur. Bl. (urinary bladder).

Table 4.2 Statistical relationships between tissue groups (continued).

TLR4

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	0	-	0	0	0	0
Lung		0	-	0	0	+	0
M. lnn			0	0	0	+	0
P. lnn				0	0	+	0
Skin					0	+	0
Spleen						0	-
Ur. Bl.							0

TLR5

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	0	0	0	0	+
Lung		0	-	-	-	-	-
M. lnn			0	0	+	0	+
P. lnn				0	0	0	+
Skin					0	0	+
Spleen						0	+
Ur. Bl.							0

TLR6

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	-	-	0	-	0	0
Lung		0	0	0	0	0	0
M. lnn			0	0	0	0	0
P. lnn				0	-	0	0
Skin					0	+	+
Spleen						0	0
Ur. Bl.							0

Key: 0 : no significant difference - $p>0.05$
+ : column tissue significantly higher than the row tissue - $p\leq 0.05$
- : column tissue significantly lower than the row tissue - $p\leq 0.05$

M. lnn (mesenteric lymph node), P .lnn (prescapular lymph node) Ur. Bl. (urinary bladder).

Table 4.2 Statistical relationships between tissue groups (continued).

TLR7

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	-	+	0
Lung		0	-	0	-	0	-
M. lnn			0	0	-	+	-
P. lnn				0	-	0	-
Skin					0	+	+
Spleen						0	-
Ur. Bl.							0

TLR8

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	0	+	+
Lung		0	+	+	-	0	-
M. lnn			0	-	-	-	-
P. lnn				0	-	0	-
Skin					0	+	+
Spleen						0	-
Ur. Bl.							0

TLR9

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	+	+	+
Lung		0	+	+	0	+	0
M. lnn			0	0	-	0	-
P. lnn				0	-	0	-
Skin					0	+	0
Spleen						0	-
Ur. Bl.							0

Key: 0 : no significant difference - $p>0.05$
+ : column tissue significantly higher than the row tissue - $p\leq 0.05$
- : column tissue significantly lower than the row tissue - $p\leq 0.05$

M. lnn (mesenteric lymph node), P .lnn (prescapular lymph node) Ur. Bl. (urinary bladder).

Table 4.2 Statistical relationships between tissue groups (continued).

TLR10

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	-	+	+
Lung		0	0	0	-	0	-
M. lnn			0	0	-	0	-
P. lnn				0	-	0	-
Skin					0	+	+
Spleen						0	-
Ur. Bl.							0

MyD88

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	0	+	0
Lung		0	0	0	0	0	0
M. lnn			0	0	-	0	0
P. lnn				0	-	0	-
Skin					0	0	0
Spleen						0	0
Ur. Bl.							0

CARD15

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	+	+	+
Lung		0	-	0	0	-	-
M. lnn			0	0	+	0	0
P. lnn				0	+	0	0
Skin					0	-	-
Spleen						0	0
Ur. Bl.							0

Key: 0 : no significant difference - $p>0.05$
+ : column tissue significantly higher than the row tissue - $p\leq 0.05$
- : column tissue significantly lower than the row tissue - $p\leq 0.05$

M. lnn (mesenteric lymph node), P .lnn (prescapular lymph node) Ur. Bl. (urinary bladder).

Table 4.2 Statistical relationships between tissue groups (continued).

CD14

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	0	+	0
Lung		0	0	+	-	-	-
M. lnn			0	+	-	-	-
P. lnn				0	-	-	-
Skin					0	0	0
Spleen						0	0
Ur. Bl.							0

Dectin-1

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	+	+	+	+	+
Lung		0	-	-	-	-	-
M. lnn			0	0	0	0	0
P. lnn				0	0	0	0
Skin					0	0	0
Spleen						0	0
Ur. Bl.							0

Dectin-2

TISSUE	Kidney	Lung	M. lnn	P. lnn	Skin	Spleen	Ur. Bl.
Kidney	0	+	-	0	0	0	0
Lung		0	-	-	-	-	-
M. lnn			0	0	+	+	0
P. lnn				0	0	0	0
Skin					0	0	0
Spleen						0	0
Ur. Bl.							0

Key: 0 : no significant difference - $p>0.05$
+ : column tissue significantly higher than the row tissue - $p\leq 0.05$
- : column tissue significantly lower than the row tissue - $p\leq 0.05$

M. lnn (mesenteric lymph node), P .lnn (prescapular lymph node) Ur. Bl. (urinary bladder).

4.3 Comparative PRR mRNA expression in ovine foetal skin and spleen

The aim of this part of this study was to determine the relative PRR mRNA expression in foetal skin and spleen compared to adult tissues. PRR mRNA expression was compared between second trimester skin and spleen with adult equivalents using two-step RT-qPCR. Foetal tissues were taken from foetuses of estimated gestational ages 60, 70, 80 and 90 and PRR expression was compared with tissues derived from healthy adult sheep.

4.3.1 Foetal Skin PRR mRNA expression

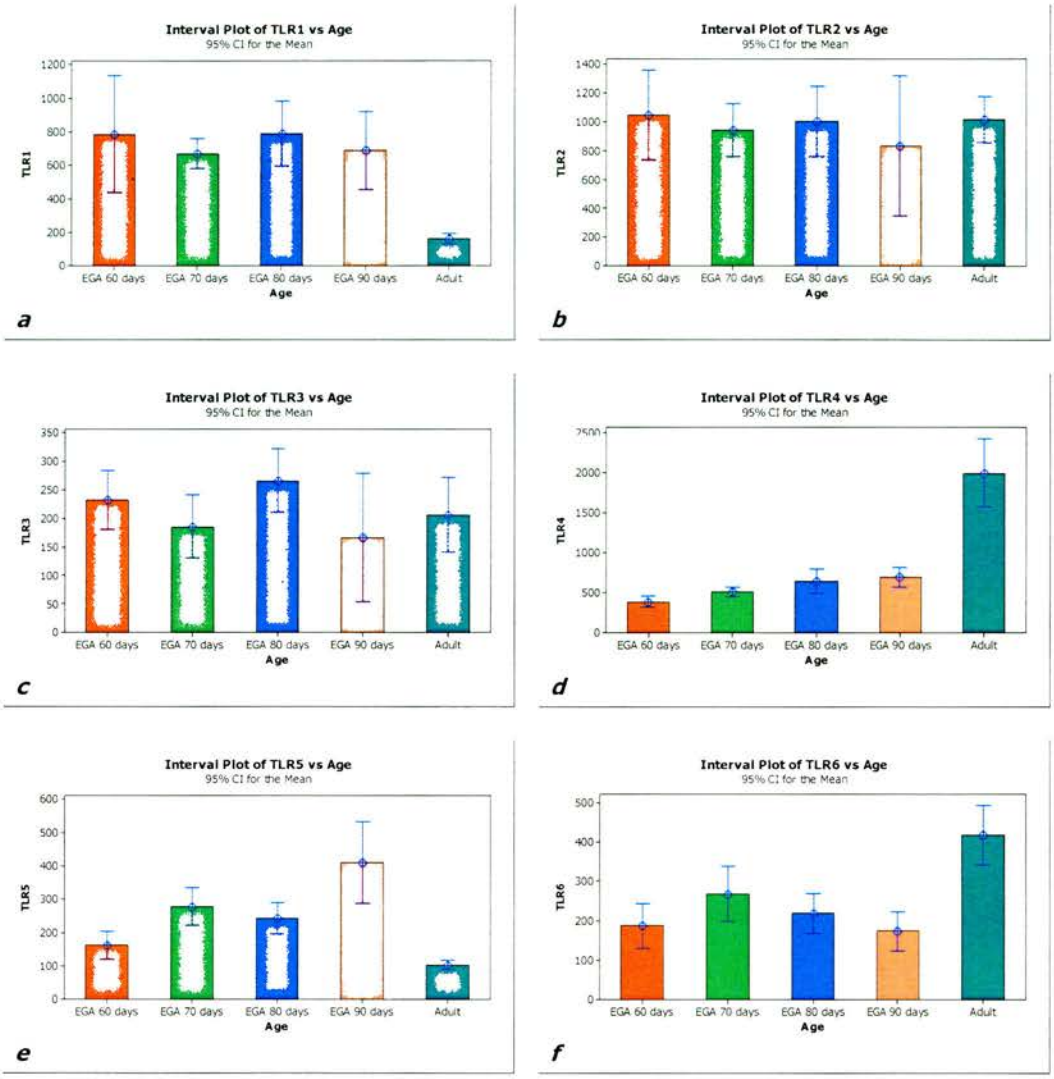


Figure 4.2 PRR mRNA expression in foetal skin

Graphical representation of TLR1 ***(a)***, TLR2 ***(b)***, TLR3 ***(c)***, TLR4 ***(d)***, TLR5 ***(e)*** and TLR6 ***(f)*** mRNA expression in foetal skin and adult skin tissue (n=5, EGA60; n=5, EGA70; n=5, EGA 80; n=2, EGA 90 and n=6, Adult). Data are expressed as bar graphs of normalized copy number per qPCR reaction of each PRR. The bars represent the mean and the whiskers representing 95% confidence interval of the mean.

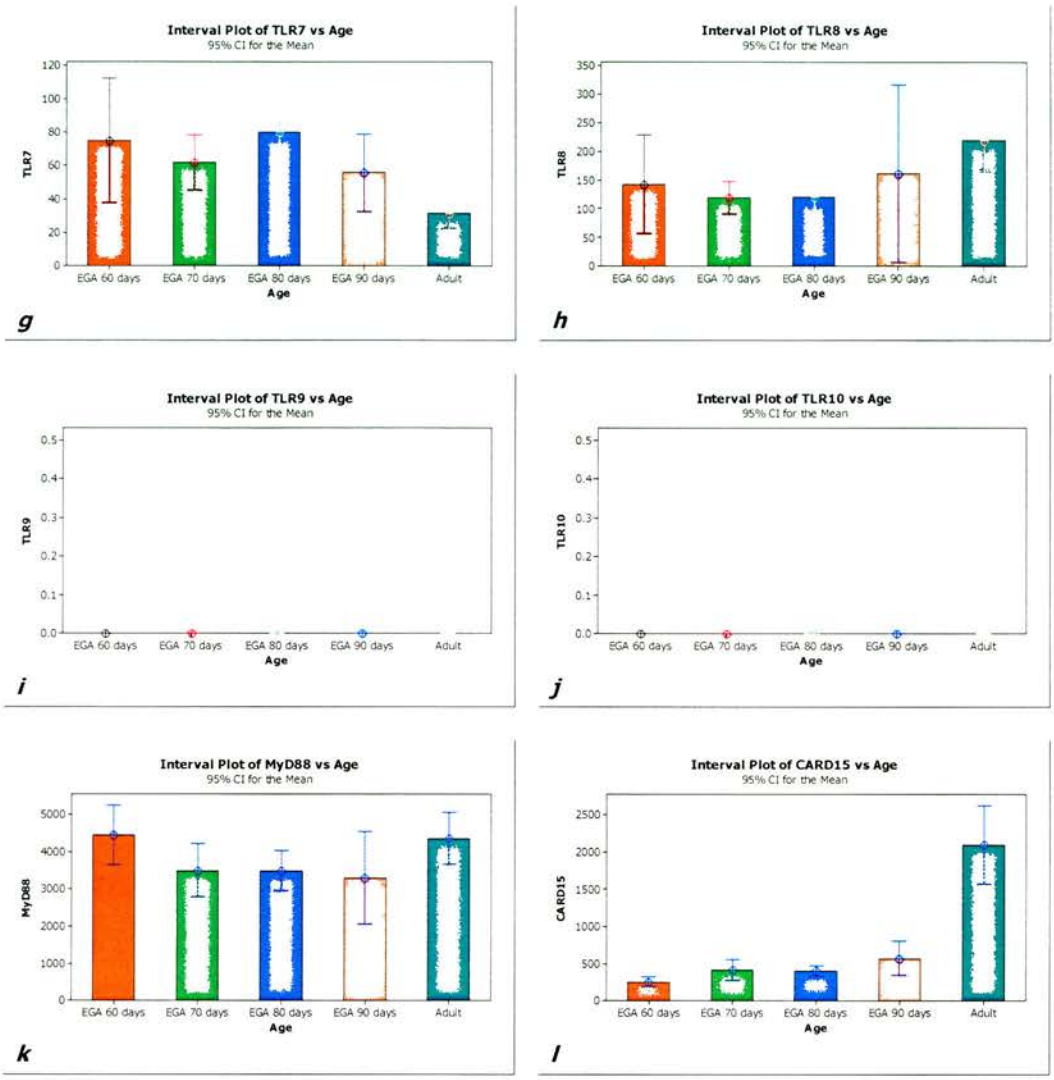


Figure 4.2 PRR mRNA expression in foetal skin (continued)

Graphical representation of TLR7 ***(g)***, TLR8 ***(h)***, TLR9 ***(i)***, TLR10 ***(j)***, MyD88 ***(k)*** and CARD15 ***(l)*** mRNA expression in foetal skin and adult skin tissue (n=5, EGA60; n=5, EGA70; n=5, EGA 80; n=2, EGA 90 and n=6, Adult). Data are expressed as bar graphs of normalized copy number per qPCR reaction of each PRR. The bars represent the mean and the whiskers representing 95% confidence interval of the mean.

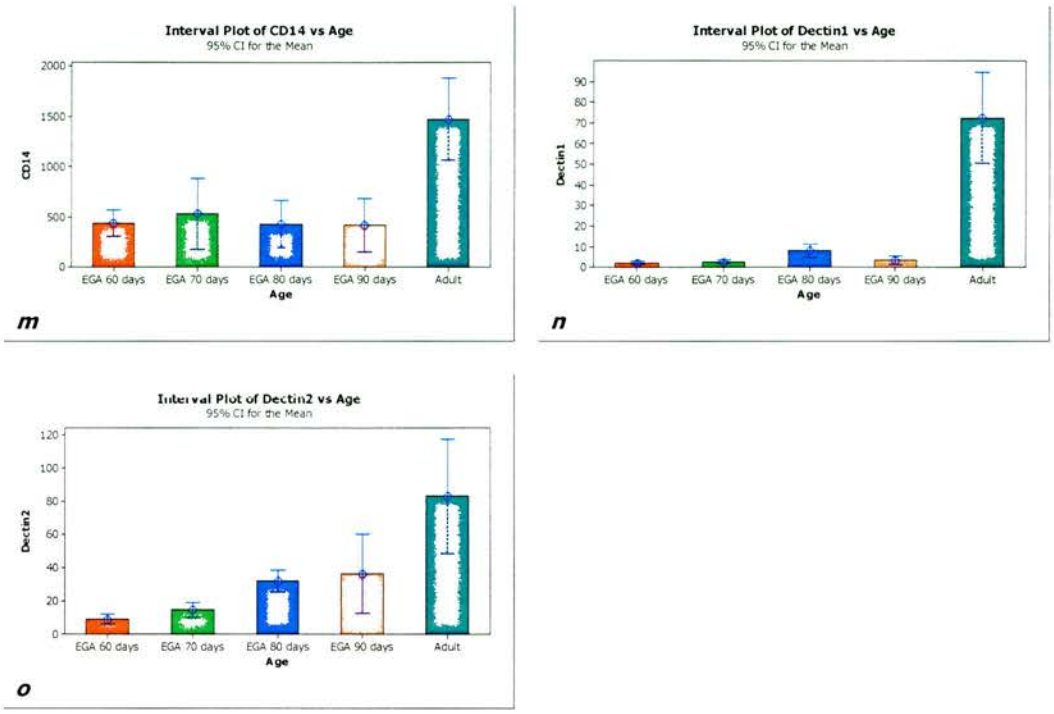


Figure 4.2 PRR mRNA expression in foetal skin (continued)

Graphical representation of CD14 ***(m)***, dectin-1 ***(n)*** and dectin-2 ***(o)*** mRNA expression in foetal skin and adult skin tissue (n=5, EGA60; n=5, EGA70; n=5, EGA 80; n=2, EGA 90 and n=6, Adult). Data are expressed as bar graphs of normalized copy number per qPCR reaction of each PRR. The bars represent the mean and the whiskers representing 95% confidence interval of the mean.

PRR expression in foetal skin

Second trimester foetal skin expresses all PRRs that are expressed in adult skin. The levels of TLR2 (Figure 4.2*b*), TLR3 (Figure 4.2*c*), TLR8 (Figure 4.2*h*), and MyD88 (Figure 4.2*k*) were not statistically significantly different between the second trimester foetal skin and the adult skin tissues. TLR9 (Figure 4.2*i*) and TLR10 (Figure 4.2*j*) were both below the lower limit of detection of the assay since only 1μg of RNA was used for the foetal tissue experiment per reverse transcription reaction.

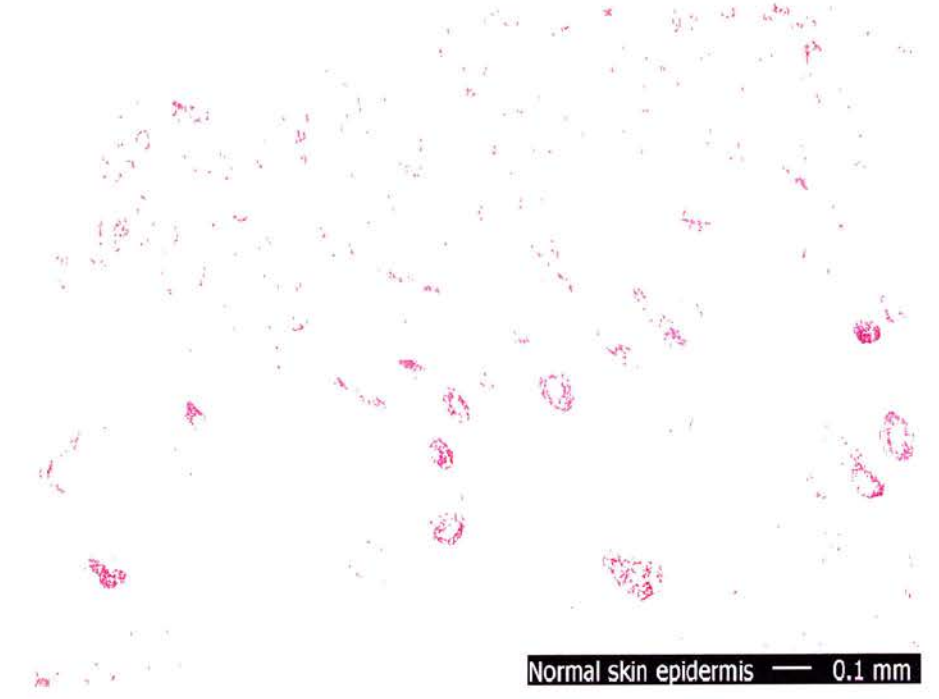
TLR1 expression in the foetal skin is similar from EGA day 60 to EGA day 90 (Figure 4.2*a*). TLR1 expression in adult skin is approximately 3.5 fold lower than the expression in foetal skin and the difference is statistically significant. TLR5 expression increases from EGA day 60 to EGA day 90 and there is a two fold increase during this gestation interval. TLR5 foetal expression is significantly different between EGA day 60 and EGA day 70 and 90 (Figure 4.2*e*), but there is no significant difference between EGA day 70 and EGA day 80. Adult TLR5 expression level is significantly lower than foetal expression during the entire second trimester period studied. TLR7 expression in foetal gestational stages is comparable. Adult TLR7 expression is approximately two fold lower than foetal expression and the difference is significant (Figure 4.2*g*),

TLR4 foetal skin expression increases steadily from EGA day 60 to EGA day 90. Adult TLR4 transcript levels are greater than three fold higher than EGA day 90 levels, and the difference is statistically significant (Figure 4.2*d*). TLR6 expression in adult skin is approximately 1.5 fold higher than second trimester foetal skin. The difference in TLR6 transcripts between adult skin and foetal skin is statistically significant but there is no significant difference in TLR6 between the gestational stages (Figure 4.2*f*). CARD15 expression in adult skin is greater than three-fold higher than foetal skin. The differences in foetal and adult skin CARD15 expression are statistically significant (Figure 4.2*l*). CD14 expression is approximately three fold higher in adult skin than foetal skin and this difference is significant (Figure 4.2*m*). There are no significant differences in CD14 expression during the gestational period EGA 60 days to EGA 90 days.

Dectin-1 expression in foetal skin ranges from imperceptible to no expression at all. Adult dectin-1 expression is also low, but is significantly higher than foetal levels (Figure 4.2*n*). Similarly, dectin-2 expression levels (Figure 4.2*o*) were significantly higher in adult skin than in foetal skin.

Figure 4.3 shows haematoxylin and eosin stained paraffin histological sections comparing EGA day 70 foetal skin and adult skin. Like the adult skin (Figure 4.3 A and C), the foetal skin shows hair follicles and a distinct epidermal layer (Figure 4.3 B, D and E). Whereas the adult skin has a more tight and dense connective tissue, the foetal skin has a looser lattice like arrangement with a moderately high cellular infiltrate. The high cellularity of the foetal skin may partially explain the comparable levels of PRRs expressed by foetal skin. The foetal skin also shows developing hair follicles.

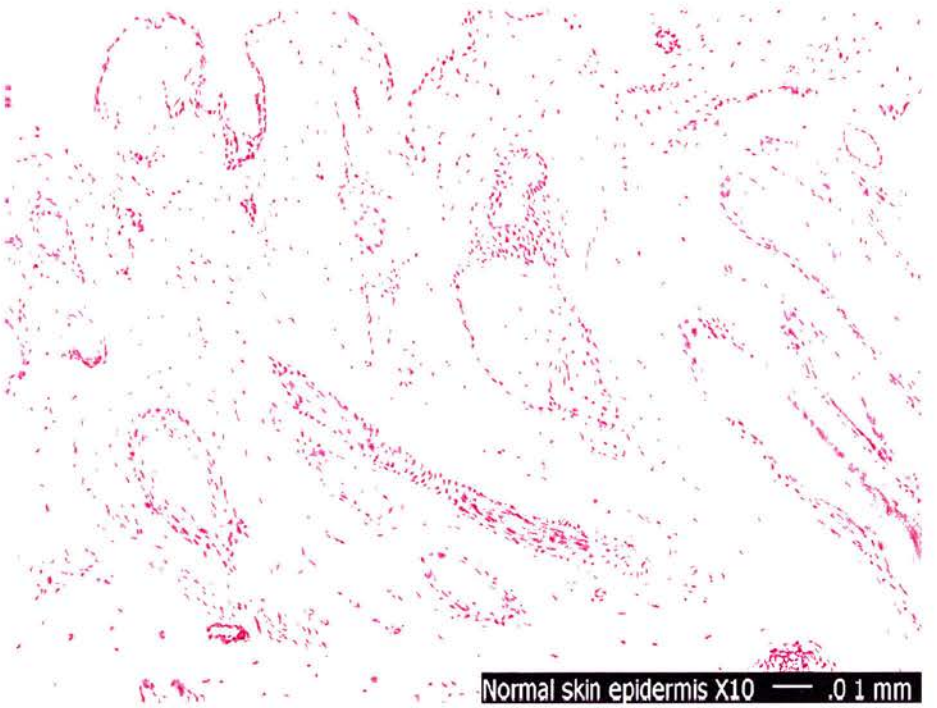
A



B

Figure 4.3 A and B

C



D

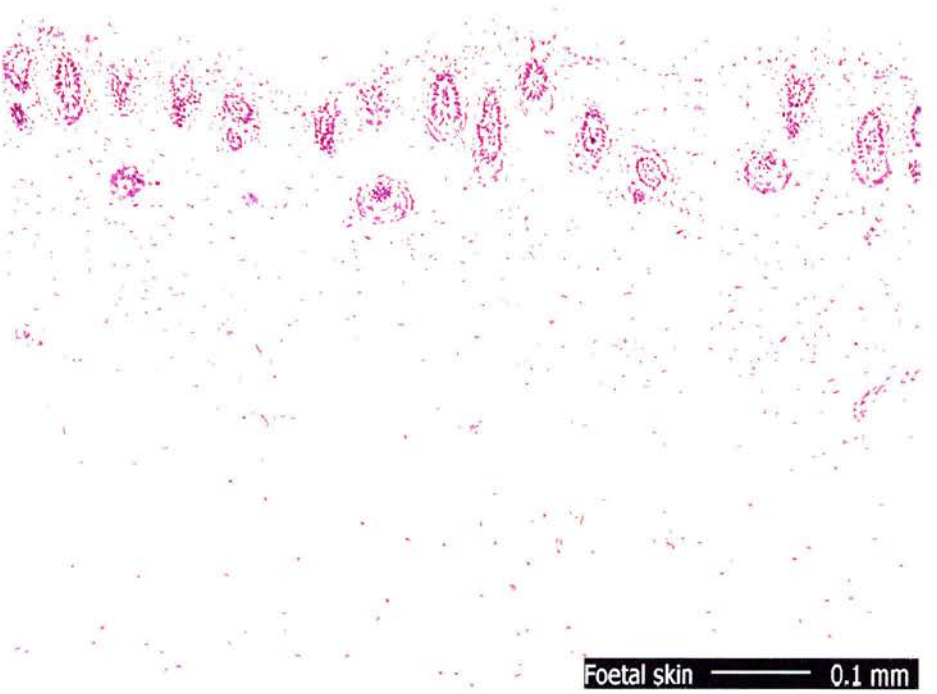


Figure 4.3 C and D

E

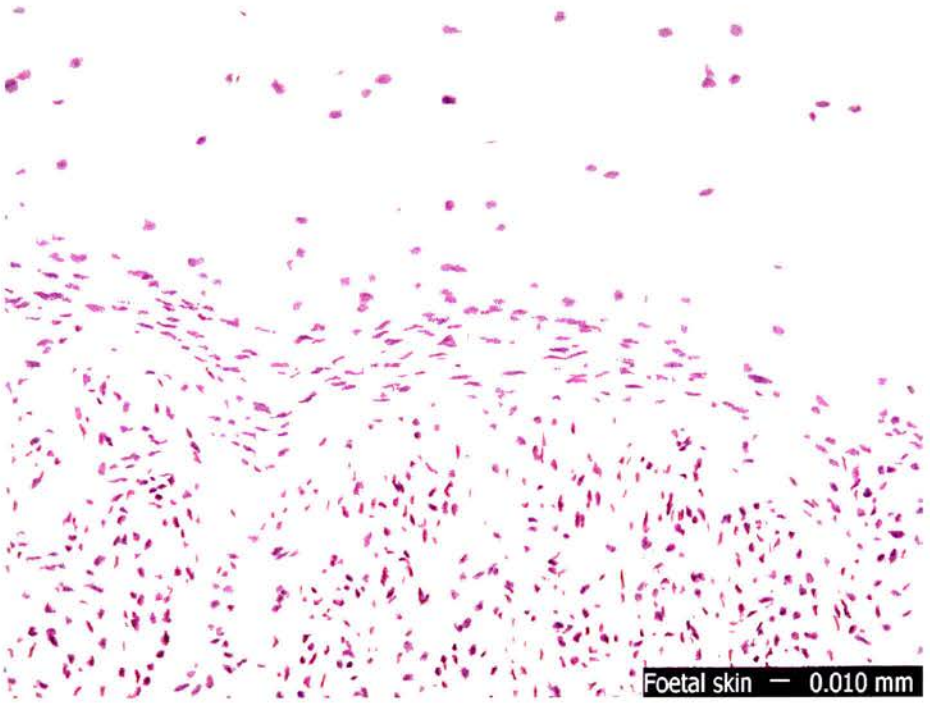


Figure 4.3 Skin architecture in adult skin and foetal skin

Haematoxylin and eosin stained paraffin section of adult and foetal skin from second trimester foetus (EGA 70) and adult ewe. Tissues fixed with zinc sulphate fixative. Figure 4.3**A** and Figure 4.3**B** 40 x magnification of adult and foetal upper skin respectively. Figure 4.3**C** and Figure 4.3**D** 100 x magnification of the epidermis of adult a foetal skin respectively. Figure 4.3**E** showing a 400 x magnification of loose lattice tissue and cellular infiltrate.

4.3.2 Foetal Spleen PRR mRNA expression

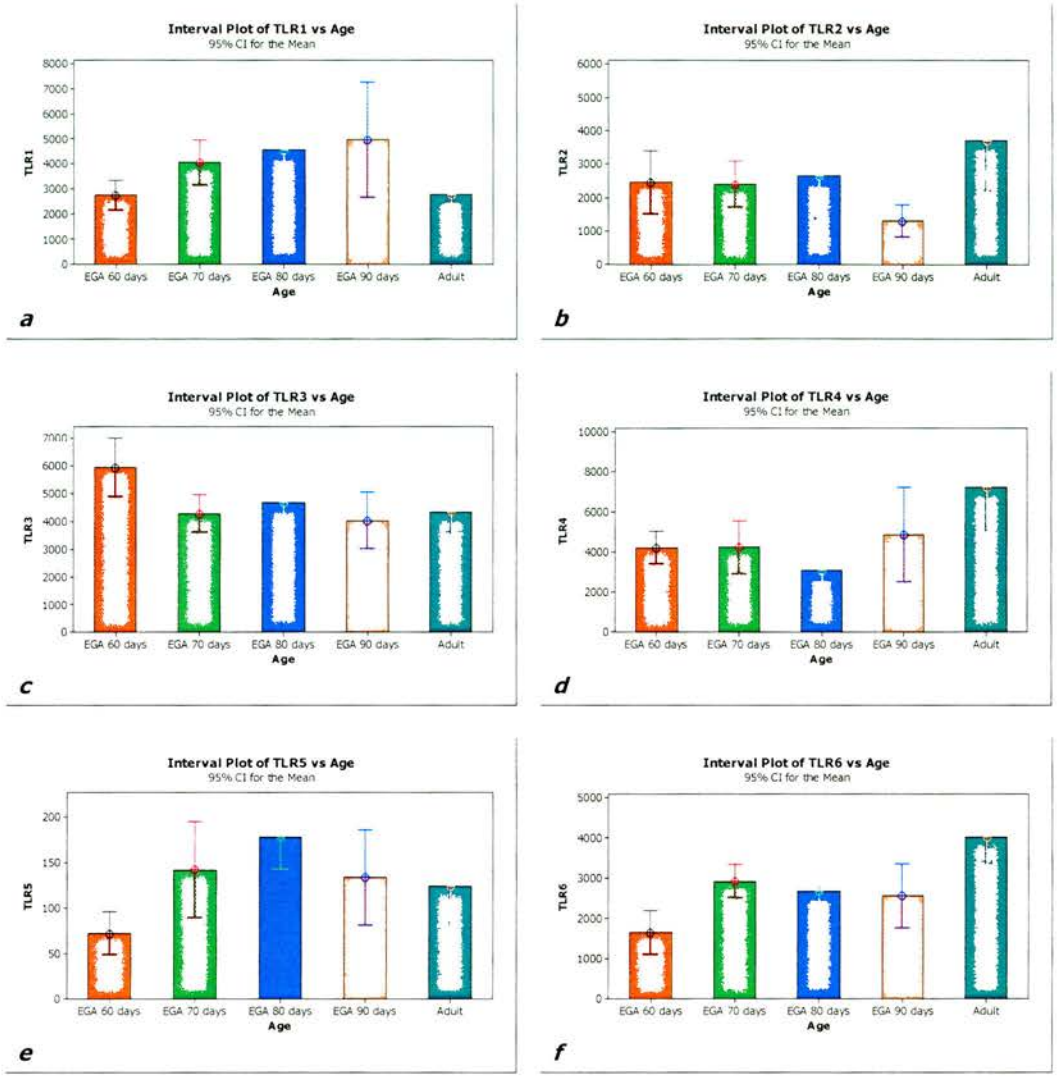


Figure 4.4 PRR mRNA expression in foetal spleen

Graphical representation of TLR1 *(a)*, TLR2 *(b)*, TLR3 *(c)*, TLR4 *(d)*, TLR5 *(e)* and TLR6 *(f)* mRNA expression in foetal spleen and adult spleen tissue (n=5, EGA60; n=5, EGA70; n=5, EGA 80; n=2, EGA 90 and n=6, Adult). Data are expressed as bar graphs of normalized copy number per qPCR reaction of each PRR. The bars represent the mean and the whiskers representing 95% confidence interval of the mean.

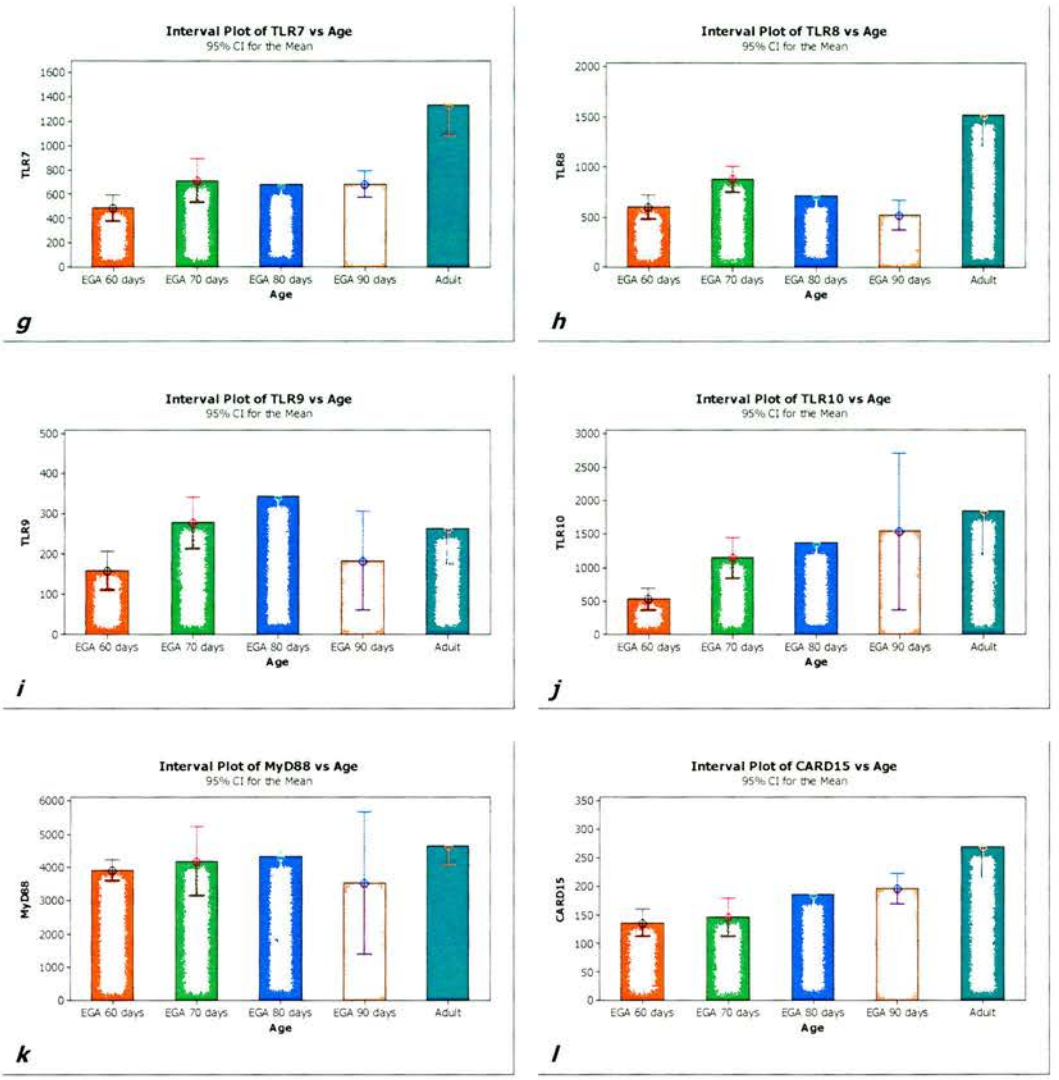


Figure 4.4 PRR mRNA expression in foetal spleen (continued)

Graphical representation of TLR7 ***(g)***, TLR8 ***(h)***, TLR9 ***(i)***, TLR10 ***(j)***, MyD88 ***(k)*** and CARD15 ***(l)*** mRNA expression in foetal spleen and adult spleen tissue (n=5, EGA60; n=5, EGA70; n=5, EGA 80; n=2, EGA 90 and n=6, Adult). Data are expressed as bar graphs of normalized copy number per qPCR reaction of each PRR. The bars represent the mean and the whiskers representing 95% confidence interval of the mean.

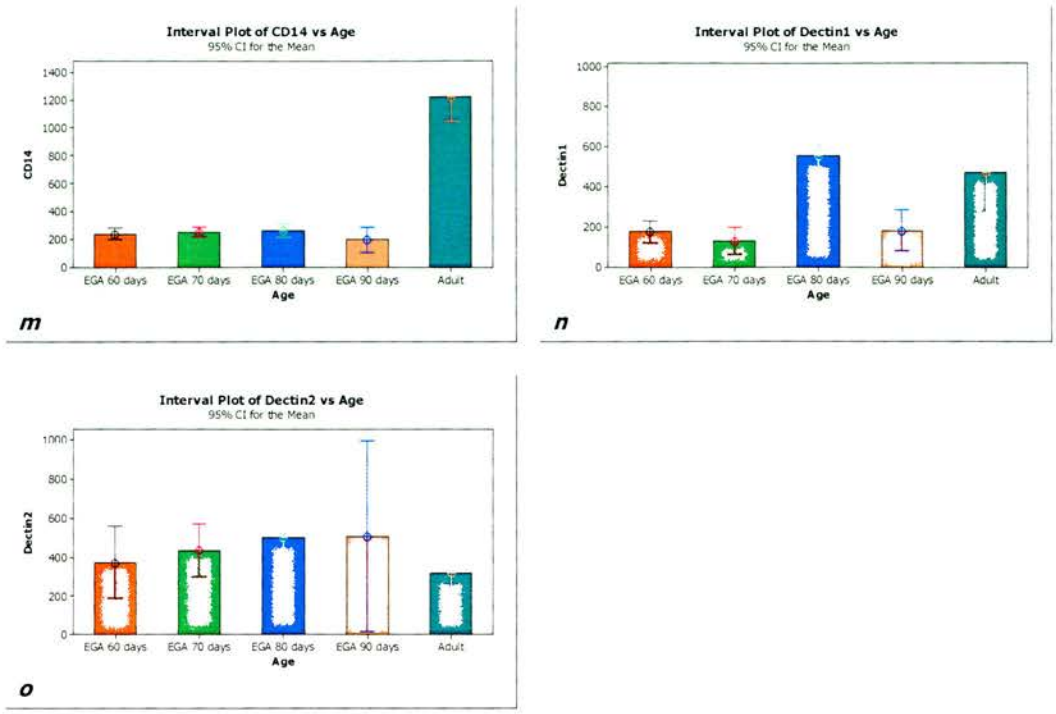


Figure 4.4 PRR mRNA expression in foetal spleen (continued)

Graphical representation of CD14 ***(m)***, dectin-1 ***(n)*** and dectin-2 ***(o)*** mRNA expression in foetal spleen and adult spleen tissue (n=5, EGA60; n=5, EGA70; n=5, EGA 80; n=2, EGA 90 and n=6, Adult). Data are expressed as bar graphs of normalized copy number per qPCR reaction of each PRR. The bars represent the mean and the whiskers representing 95% confidence interval of the mean.

PRR expression in foetal spleen

Similar to foetal skin, foetal second trimester spleen expressed all PRRs that are expressed in adult spleen.

TLR1 expression in the foetal spleen increases from EGA day 60 to EGA day 90. There is a significant difference in TLR1 expression between EGA day 60 and EGA day 80. Adult spleen TLR1 expression is lower than foetal EGA day 70 to EGA day 90, but the difference is not significant (Figure 4.4*a*). TLR2 expression in the foetal spleen is similar from EGA day 60 to EGA day 80 and these levels are comparable to adult TLR2 transcript levels. EGA day 90 spleen has a lower TLR2 expression than the other foetal stages (Figure 4.4*b*). TLR3 expression in the foetal spleen is similar from EGA day 70 to EGA day 90 and these levels are comparable to adult TLR3 transcript levels (Figure 4.4*c*). EGA day 60 spleen has a higher TLR3 expression than the other foetal stages but the differences are not significant. Adult spleen has a slightly higher expression of TLR4 than foetal second trimester spleens, but these differences are not statistically significant (Figure 4.4*d*). TLR5 expression increases in foetal spleen from EGA day 60 to EGA day 80 and there is a slight drop in expression levels at EGA day 90 (Figure 4.4*e*). Adult levels of TLR5 are similar to EGA day 90 levels. The differences in TLR5 expression during the second trimester and adult levels are not significant. Adult TLR6 expression is slightly higher than foetal expression and this difference is statistically significant. EGA day 60 spleen has an approximately two-fold lower expression than the other gestational stages and this difference is statistically significant (Figure 4.4*f*).

Adult spleen had an approximately two fold higher expression of TLR 7 than foetal spleen (Figure 4.4*g*) and this difference is significant. There was no significant difference in spleen TLR7 expression within the second trimester gestational stages. Similarly, adult spleen had a greater than two fold higher expression of TLR 8 than foetal spleen (Figure 4.4*h*) and this difference is significant. There was also no significant difference in spleen TLR8 expression within the second trimester gestational stages.

TLR9 expression increases in foetal spleen from EGA day 60 to EGA day 80 and there is a drop in expression levels at EGA day 90 (Figure 4.4*i*). There is a significant difference in TLR9 transcripts between EGA day 60 and EGA day 80. The differences in TLR9 expression during the second trimester spleen and adult levels are however not significant.

TLR10 shows a steady increase from EGA day 60 to adult levels. EGA day 60 has significantly lower expression of TLR10 than EGA day 70, EGA day 80 and adult spleen levels (Figure 4.4*j*). Adult spleen TLR10 expression is however, not significantly different with EGA day 70, EGA day 80 and EGA day 90 spleen.

MyD88 expression is comparable between foetal spleen and adult spleen (Figure 4.4*k*).

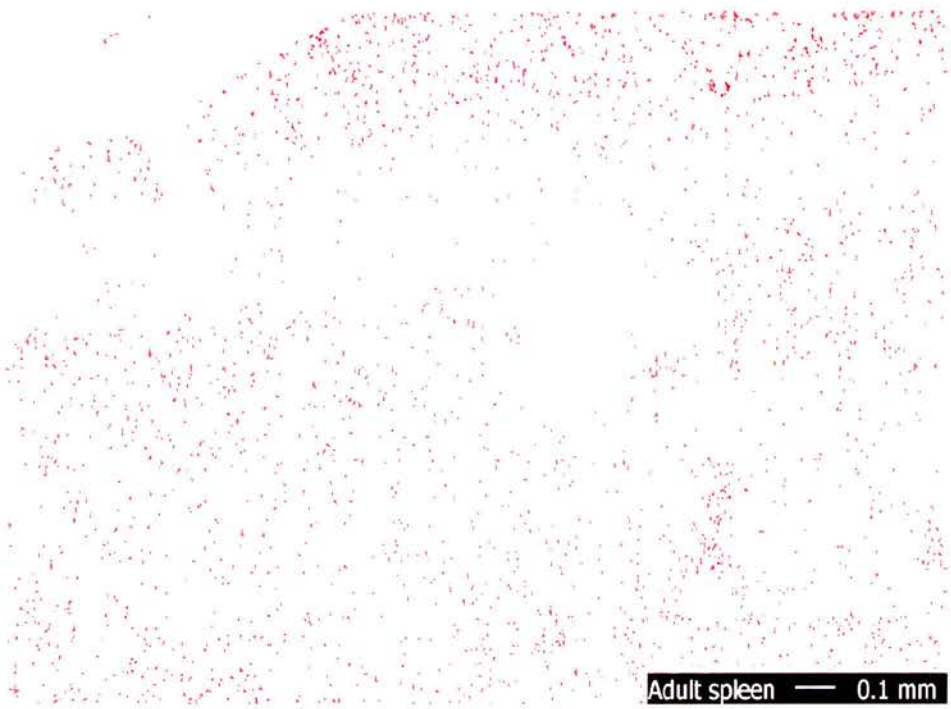
CARD15 shows a steady increase from EGA day 60 to adult levels. EGA day 60 has significantly lower expression of CARD15 than EGA day 90 and adult spleen levels. Adult spleen CARD15 expression is also significantly higher than EGA day 70 spleen, but there is no significant difference with EGA day 80 and EGA day 90 spleen (Figure 4.4*l*).

CD14 had an approximately 5 fold greater expression in adult spleen than foetal spleen (Figure 4.4*m*) and this difference is statistically significant. There is no significant difference in CD14 expression between the gestational stages spleens.

Dectin-1 and dectin-2 do not show any statistically significant differences in expression between adult spleen and foetal second trimester spleen (Figure 4.4*n* and Figure 4.4*o*).

Figure 4.5 shows haematoxylin and eosin stained paraffin histological sections comparing EGA 70 foetal spleen and adult spleen. The sections show the normal lymphoid aggregations of adult spleen and in comparison shows the commencement of such aggregations and in some instance taking up adult splenic architecture where the aggregations are around a central arteriole.

A



B

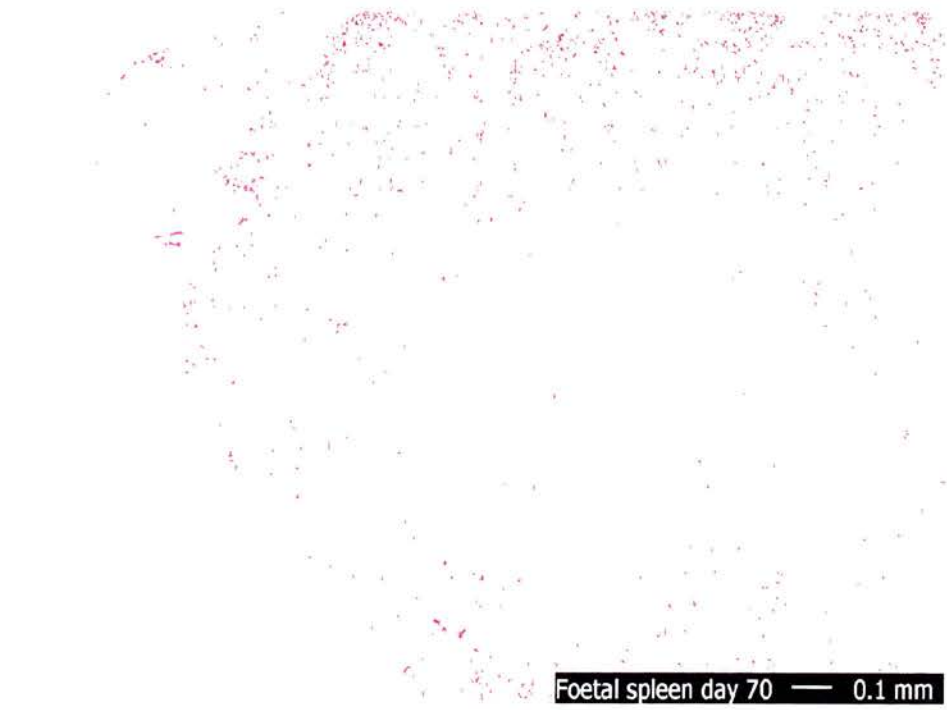
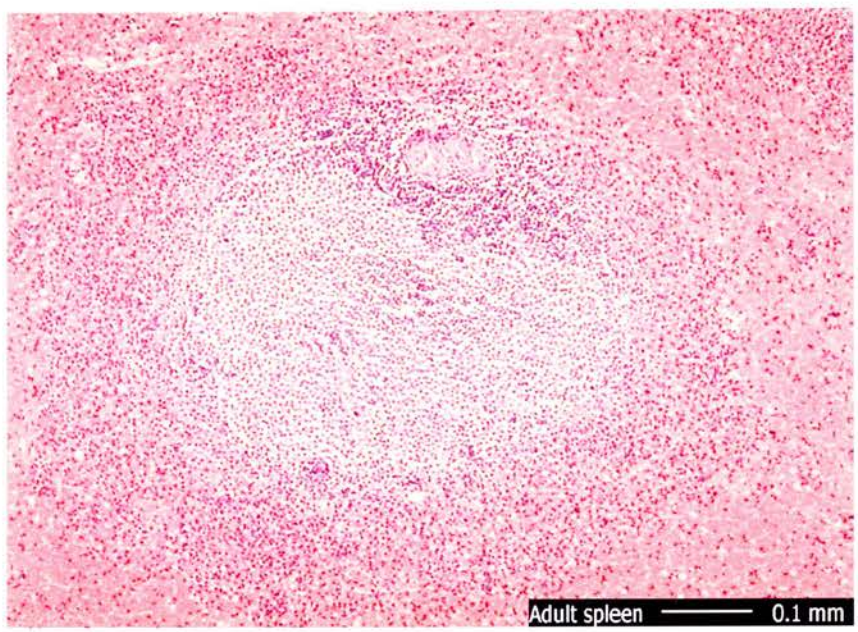


Figure 4.5 A and B

C



D

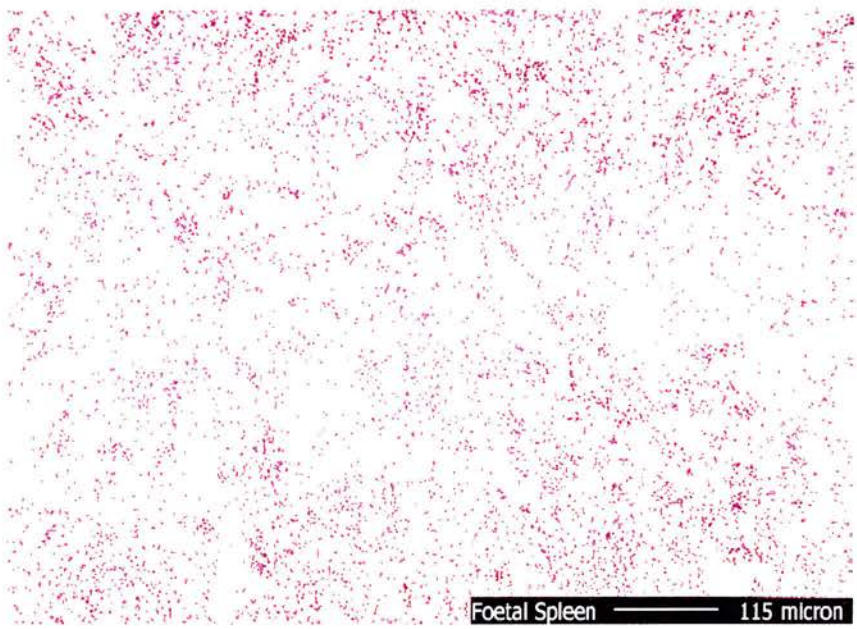


Figure 4.5 Splenic architecture in adult and foetal spleen

Haematoxylin and eosin stained paraffin section of spleen from adult ewe and second trimester foetus. Tissues fixed with zinc sulphate fixative. Figure 4.5 **A** and **B**, 40 x magnification of adult and foetal spleen respectively. Figure 4.5 **C** and **D** 100 x magnification of adult and foetal spleen respectively. In Figure 4.5 **C** the adult spleen showing the development of organised lymphoid aggregations. These aggregations are organized around a central arteriole and similarly the foetal spleen shows the commencement of such an organizational pattern.

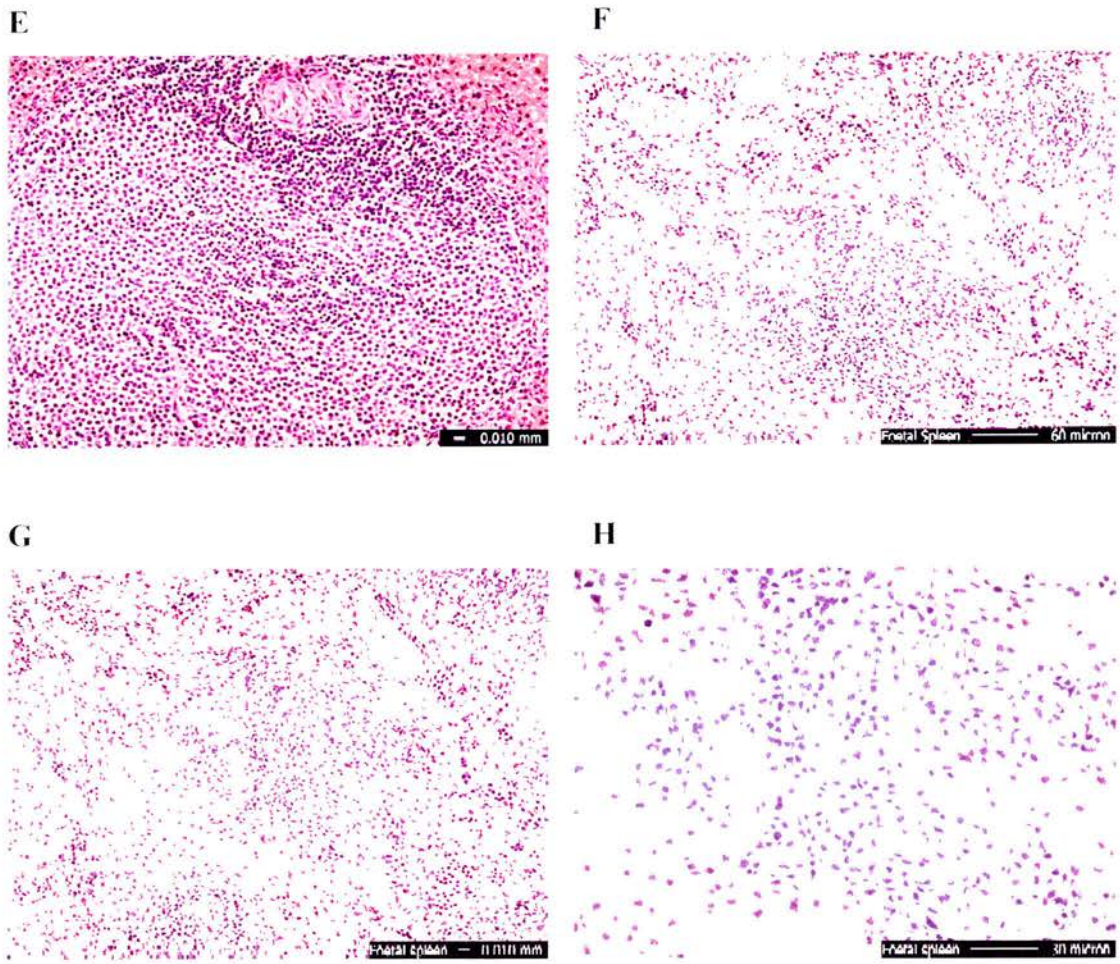


Figure 4.5 Splenic architecture in adult and foetal spleen (continued)

Haematoxylin and eosin stained paraffin section of spleen from adult ewe and second trimester foetus. Tissues fixed with zinc sulphate fixative. Figure 4.5 **E** and **F**, 200 x magnification of adult spleen follicle and foetal follicular aggregation respectively. Figure 4.5 **G** 200 x magnification of foetal spleen follicle cell aggregation. Figure 4.5 **H** 400 x magnification of foetal spleen follicle cell aggregation showing the composition of the cellular infiltrate.

4.4 Discussion

The innate immune system recognizes phylogenetically conserved motifs on pathogens referred to as PAMPs and also endogenous products (referred to as 'danger signals') released during tissue injury via PRRs. During the last decade, much progress has been made in unravelling the complex role that PRRs play in PAMP recognition and immune modulation but many questions still remain to be answered.

The aim of this part of this study was to determine the expression and distribution of particular PRRs and MyD88 in foetal tissues (skin and spleen) and selected adult tissues (skin, spleen, kidney, lung, mesenteric lymph node, prescapular lymph node and urinary bladder) from normal healthy subjects. Quantitative real time PCR assays were developed and optimized for the PRR panel and MyD88 and normalized to the housekeeping genes β-actin, and SDHA. The quantitative real time PCR assays revealed reliable and consistent amplification of target genes within the tissues examined. The preceding data shows the PRR mRNA expression diversity in the adult ovine tissues and also a comparison between PRR mRNA expression in second trimester spleen and skin and the respective adult tissues.

The kidney had a low to moderate PRR expression compared to other tissues studied except for TLR4 and TLR6 where the kidney expression was on the higher end. PRR expression levels of the kidney were generally slightly lower or comparable to the expression levels of the urinary bladder (which together form the major parts of the urinary system). This may reflect the fact that the kidney has a lower contact with antigens/pathogens due to its anatomical location and lack of direct contact with the external environment. The kidney is an internal organ that is uncommonly infected in the sheep and it does not normally harbour resident commensal microflora. This could explain the low PRR expression observed in the kidney by this study. The urinary bladder contains urine, a waste product of the body, and the urine may contain immunostimulatory factors. Uric acid has been shown to be an endogenous ligand of TLR2. This study showed that the urinary bladder had a very low expression of TLR2, and I speculate that this may be a homeostatic mechanism to

prevent a hyper-inflammatory state that would arise if there were a raised TLR2 mediated immune responses to urates in the urine. This would be a mechanism similar to the one observed in the gastrointestinal tract mucosae with LPS sensing PRRs, TLR4 and CD14, which have very low expression to prevent hyperinflammatory responses of the gut to commensal organisms that also possess LPS (Abreu *et al.*, 2001; Smith *et al.*, 2001; Smythies *et al.*, 2005).

The lung generally had very high PRR expression levels. This may reflect the high pathogen/antigen stimulation it receives at its mucosal surfaces due to its anatomical location and intrinsic physiological function of air exchange. Air often contains low levels of microbes and particulate material that would inevitably keep the lungs constantly stimulated. The lung had a high expression of TLR2, a PRR necessary for the detection of Gram positive bacteria and mycobacteria. High TLR2 expression has been specifically demonstrated in human alveolar macrophages and lung epithelial cells (Droemann *et al.*, 2003), cells that are important in initial pathogen contact and sensing in the lung. The TLR2 expression from these cell types could contribute to the high TLR2 expression seen in the ovine lung in this study. The lung also had the highest expression of dectin-1 and dectin-2; findings which are consistent with findings reported by Brown and Gordon (Brown and Gordon, 2001) for dectin-1 in humans but different from the findings reported by Bonkobara and colleagues (Bonkobara *et al.*, 2006) who found that the lymph nodes had the highest expression of dectin-2 in cattle. Willcocks and colleagues (Willcocks *et al.*, 2006) showed a consistent dectin-1 expression in alveolar macrophages and the high number of these cells in the lung could contribute to the high expression observed. Gavino and co-workers (Gavino *et al.*, 2005) also found a high expression of human dectin-2 in the lung (amongst the non-lymphoid tissues) and and unlike this study found no expression in the kidney. Amongst lymphoid tissues, Gavino and co-workers (2005) found that the spleen had a higher dectin-2 expression than lymph nodes. This study showed comparable expression of dectin-2 between all the other tissues except the lung that showed a seven to ten-fold higher difference in expression as compared to the other tissues. In contrast to findings by Ariizumi and colleagues (Ariizumi *et al.*, 2000), who found highest expression of dectin-2 in mouse spleen and very low

expression in skin, urinary bladder and kidney. The lung also had a high expression of TLR5, and this finding agrees with the data of Sebastiani and co-workers (Sebastiani *et al.*, 2000) and Zarember and Godowski (Zarember and Godowski, 2002). Similar to the findings of Nishimura and Naito (Nishimura and Naito, 2005) this study showed that the lung and urinary bladder had comparable expression of TLR5 and similarly, that expression was higher than the expression in the spleen. TLR5 expression is hypothesized to play an important role in the innate immune defence mechanisms of the lung against inhaled flagellated bacteria since a mutation in TLR5 has been associated with increased susceptibility to Legionnaires disease (Hawn *et al.*, 2003).

A rather surprising finding from this study is the low relative mRNA expression of most PRRs by the skin (except for TLR4, TLR5, CD14, dectin-1, and dectin-2 where levels were comparably moderate). One would expect that being constantly exposed to the environment, the skin would have significantly higher amounts of PRRs compared to other tissues reflecting that constant contact with a pathogen filled environment. Using semi-quantitative PCR, Ishii and co-workers (Ishii *et al.*, 2006) showed a lower expression of TLR2 in canine skin and urinary bladder compared to the lung and spleen. This is in concordance with the findings of this study. This may reflect the fact that the immune system in the skin is effectively separated from the externum by the impervious keratinized squamous cell layer. Thus, the low PRR expression may indicate the resulting low immunological activation of the steady state cellular constituents of skin and also its capability to recruit inflammatory cells from the bloodstream during times of need. Liu and co-workers (Liu *et al.*, 2003) also found a low/absent expression of TLR9 in mouse skin but a capacity to increase expression during physical trauma attributed to the recruitment of TLR9 expressing blood leucocytes. Another possible way of looking at the low expression of PRRs in the skin is that it could be an immune tolerance mechanism (similar to that found in the gut), since the skin is also quite well endowed with commensal organisms and would then otherwise be in a constant state of inflammation. It may thus represent a specialized mechanism for retaining the capacity for selectively responding to pathogens while maintaining homeostatic tolerance to resident commensal

organisms. Other groups working with innate immune mechanisms have demonstrated the low level expression of certain PRRs and hyporesponsiveness of these epithelial surfaces to PAMPs (Abreu *et al.*, 2001). Similar immune mechanisms may not be suitable for other tissues that may need to mount a robust immune response to any microorganism that may invade it, including microbes that may reside as commensals elsewhere. This fact is aptly demonstrated by the fact that commensal/symbiotic organisms that are beneficial in one tissue may be harmful and cause disease in another tissue of the same host. The fact that these specimens were collected from indoor sheep could also reflect the low level of pathogenic challenge on the skin as compared to outdoor reared or feral sheep. CARD15 however, was consistently highly expressed in skin than any other tissue. I speculate that the high CARD15 expression in the skin could arise from the Langerhans cells (LC) and other dermal APCs. This is based my extrapolation from work by Ginhoux and co-workers (Ginhoux *et al.*, 2006) who show that LCs are derived from blood monocytes, and cells of the macrophage/monocyte lineage are known to highly express CARD15 (Gutierrez *et al.*, 2002).

This study demonstrated that the lymph nodes and spleen generally had higher expression of most PRRs compared to the other tissues examined. Lymphoid tissues are regions of high infiltrate of immune cells that are activated from the periphery and resident immune cells. This may have a bearing on their high PRR expression observed in the lymphoid tissues studied. Ochoa and colleagues (Ochoa *et al.*, 2003) were able to demonstrate the co-localization of TLR2 expression with DCs and macrophages in human secondary lymphoid tissues. Ochoa and colleagues (Ochoa *et al.*, 2003) were however not able to detect TLR1 or TLR2 on B cells and T cells in these secondary lymphoid organs although the TLR1 and TLR2 expressing APCs were closely apposed to these cells.

Lung and all lymphoid tissues had a very high expression of TLR7 compared to skin and urinary bladder. These findings are similar to those by Philbin and co-workers (Philbin *et al.*, 2005) who also speculates that the high immune cell content could explain the high TLR7 in these tissues. These findings on TLR7 expression in lung and spleen also concur with the findings of Nishimura and Naito (2005). This study

demonstrated higher levels of TLR10 in the lung, mesenteric and prescapular lymph nodes and imperceptible levels in the skin and kidney. This is in agreement with the findings of Chuang and Ulevitch (Chuang and Ulevitch, 2001) who found high expression levels in human lymphoid tissues and the findings of Nishimura and Naito (2005) who found high TLR10 expression in the spleen and lung. The high expression of TLR10 in the lymph nodes and spleen may reflect the abundance of APCs cells such as B cells and DCs that are reported to express high quantities of TLR10 (Hasan *et al.*, 2005). Similar to the findings of Nishimura and Naito (2005) the spleen expressed the highest amount of TLR4 compared to other tissues. The relative proportions of TLR8 in the spleen and the mesenteric lymph nodes is similar to those recorded by Ignacio and co-workers (Ignacio *et al.*, 2005) in cats. Mesenteric and prescapular lymph nodes expression of PRR was statistically comparable for all PRRs studied except for TLR8 and CD14, where mesenteric lymph nodes had a significantly higher TLR8 expression than prescapular lymph nodes but a significantly lower CD14 expression. Expression of the TLR adaptor molecule, MyD88, between these two lymph nodes was comparable. Similar to this study, Hashimoto and co-workers (Hashimoto *et al.*, 2005) also found the expression of TLR9 in canine spleen and lymph nodes, but unlike this study did not detect any TLR9 in the skin, bladder and kidney. This study found lower expression in skin, bladder and kidney and the difference could be ascribed to the lower sensitivity of the qualitative RT-PCR assay they used compared to the quantitative real time PCR used in this study. The CARD15 expression from this study is similar to that found by Iwanaga and colleagues (Iwanaga *et al.*, 2003) in mice with regards the relative expression of CARD15 in the spleen, lung and kidney. Iwanaga and colleagues (2003) found that the lung had a two-fold higher CARD15 expression than the spleen and about eight-fold higher than the kidney.

TLR4, TLR6 and MyD88 were the only genes that did not have substantial tissues variation greater that five fold between the different adult tissues and from this study I conclude that TLR4, TLR6 and MyD88 are expressed ubiquitously in the tissues studied.

Recently, Menzies and Ingham (Menzies and Ingham, 2006) published findings on TLR expression in sheep. Some of my findings are in contrast to their findings although in that study only three ovine tissues were looked at, namely Peyers patch, jejenum, mesenteric lymph node, and the bovine skin. In the bovine skin they found that TLR7 was the most abundant transcript, I found that TLR7 was mostly expressed in very low levels and the expression was similarly low in foetal skin. The presence of low levels of TLR7 in skin is similar to that found by Philbin and co-workers (Philbin *et al.*, 2005) in chickens. In concordance with the findings of Menzies and Ingham (2006) I also found TLR 9 and 10 being very lowly expressed in skin but unlike their finding, where they found an absence of TLR6 this study revealed moderate amounts of TLR6 in adult skin and also in foetal skin. The findings on low cutaneous TLR9 expression in the present study also agree with the findings of Hashimoto and co-workers (Hashimoto *et al.*, 2005) who were unable to detect TLR9 in canine skin using qualitative RT-PCR. The differences with the skin PRR expression could be a species difference considering that their study looked at TLR expression in bovine skin and did not examine TLR expression in ovine skin. They also found a higher expression of TLR2 in bovine skin which is different from the findings of this study and that of other studies looking at TLR2 in human skin and canine skin (Ishii *et al.*, 2006).

The tissues' specific physiological mRNA expression profiles of the different PRRs may reflect the most likely pathogens (or PAMPs) each tissue is likely to encounter and its relative preparedness for the challenge. On the other hand it may also represent the actual steady state 'PAMP load' of each tissue since PRRs are known to sense endogenous PAMPs such as heat shock proteins that may reflect the normal physiological tissue destruction and re-modelling. Mucosal surfaces are colonised by commensals and have a higher likelihood of encountering pathogens compared to internal organs that are considered 'sterile'. They thus need to be able to distinguish between commensal and pathogens and thus generate homeostatic or protective immune responses respectively.

Different tissues also seem to have a predisposition towards a particular class of effector immune response that are often quite polarized. Delayed type

hypersensitivity characterized by the hallmarks of inflammation, as described by Cornelius Celsus (30BC to 38AD), redness, heat, pain, swelling, is more likely to occur in the skin on exposure to antigen. The obverse is true with tissues such as the gut, eye and brain where such an immune response would be catastrophic. In such tissues antigenic stimulation thus tends to favour Th2 responses predominated by antibody production to protect the tissues from self damage. For example, if the gut were to have such an excessive inflammatory response, it would expose the sub-epithelial tissues to the dense gut microbiota and this would have severe consequences.

In conclusion, the study of PRR expression in healthy adult tissues shows that, consistent with their ability to mount immune responses, all tissues expressed the studied PRRs, though individual tissues had different PRR expression profiles/patterns. Secondary lymphoid tissues and lung mucosae generally have higher PRR expression, compared to the other tissues studied. Some PRRs showed great variations in expression in the same tissue and this is most likely due to the inherent variability that is to be found between these outbred sheep compared to experimental inbred mouse strains. Larger population studies would elucidate such potential expression differences.

Ascribing rigid functional limitations on the capacity of a tissue to respond to a particular class of pathogens based on tissue PRR expression must be done with caution as it may be erroneous in the absence of more extensive studies on PRR expression of a tissue's constituent cells in relation to cellular composition. This is particularly so, because it is known that the cellular composition of a tissue has a direct translation to its PRR expression and immune capabilities. Further, tissues are also able to recruit immune cells such as monocytes from blood that may rapidly alter overall tissue PRR expression by modifying tissue cellular proportions. This is characteristically evident during healing, infection and inflammation. Studies in the dynamics of tissues recruiting such cells in physiological and pathological situations in relation of PRR expression may be quite revealing and provide useful insights of the real time immune capacity of tissues. Functional studies in tissue PRR expression would be further strengthened by concurrent proteomic analysis and

immunohistological analysis of tissues to identify immune cell populations. These options are however currently not possible due to the absence of specific monoclonal antibodies to most of these PRRs. A single ligand may also stimulate multiple PRRs and each PRR may be able to compensate for the other. This is typically exemplified by viral RNA recognition by TLR3 but also by RIG-1 and MDA5 where either or all PRRs may be engaged during viral infection. Although possibly of minor importance, the presence of spliced variants that have now been described for numerous PRRs (Ariizumi *et al.*, 2000; Leung *et al.*, 2007; Philbin *et al.*, 2005) may account for some differences that may be seen in quantified mRNA expression. Spliced variants have been shown to be tissue specific although the functional implication on immune function has not been elucidated. Where spliced variants have been described, comparative quantification of the expression of the variants in a tissue would be of value in future experiments.

PRR expression in preterm skin and spleen

Very few data are currently available on the molecular immune mechanisms in foetuses of higher mammals such as ruminants or humans. Ethical considerations have hampered this research in humans and ethical - economical considerations in veterinary species. The lack of absolute differences in most of the second trimester PRR expression in spleen and skin as compared to adult equivalents shown in this study was rather surprising. Since it is a documented fact that neonates are more susceptible to bacterial infections especially, one would assume that PRRs being placed so central in innate immunity would have some correlation to this increased susceptibility.

The skin generally has low levels of TLR1 and surprisingly adult skin expressed significantly lower TLR1 compared to second trimester foetal skin. This may be due to some stimulation of the foetal skin that may arise from its constant contact with amniotic fluid. Similarly, TLR5 and TLR7 levels were lower in adult skin than foetal skin. Foetal skin has imperceptible levels of TLR9 and TLR10 mRNA. The levels were below the level of quantification but weak bands were visible on gel electrophoresis. This was particularly evident with the foetal tissue experiment where 1μg of RNA was used for the RT reactions compared to the 2.5μg in the adult tissues experiment. TLR9 has been shown to be very lowly expressed or absent in mammalian skin (Liu *et al.*, 2003). Expression of TLR2, TLR3, TLR8 and MyD88 were comparable between second trimester foetal skin and adult skin. Expression of TLR4, TLR6, CARD15, CD14, dectin-1 and dectin-2 were significantly higher in adult skin than foetal skin. CARD15, CD14, dectin-1 and dectin-2 are known to be highly expressed in cells of myeloid origin and the significant differences in expression of these PRRs may reflect the differences in the composition of myeloid derived cells in adult and foetal skin.

Histology of EGA day 70 foetal skin shows that it is well interspaced with numerous cells. The high cutaneous cell content could translate to high PRR expression since many cells of the skin such as keratinocytes, Langerhans cells, fibroblasts and dermal DCs are known to express PRRs (Kollisch *et al.*, 2005). Foetal skin histology also

showed that the skin has numerous developing hair follicles. Pivarcsi and colleagues (Pivarcsi *et al.*, 2003) showed that hair follicles have a high expression of TLR2 and TLR4. This may explain the comparable level of foetal TLR2. The TLR4 in adult skin was however, significantly higher than foetal skin.

Ligation of PRRs has been shown to have a role in the release of antimicrobial peptides such as α and β-defensins (Sumikawa *et al.*, 2006; Vora *et al.*, 2004). The significant level of PRRs expressed in foetal skin could also play a role in the formation of the *vernix caseosa* present on foetal skin at birth and possessing antibacterial properties. Antigenic stimulus may arise from the amniotic fluid in which the foetus is suspended and encourage the formation of the protective layer.

The spleen is the largest lymphoid tissue accounting for about one quarter of lymphocytes in humans. Ovine foetal spleen has comparable mRNA expression to adult spleen for most of the PRRs studied except for TLR7, TLR6 and CD14, in which the adult has significantly higher mRNA expression. This may be direct proof that the foetal spleen (and thus the innate immune system) is almost fully mature in second trimester sheep foetuses. Work done by Wilson and co-workers (Wilson *et al.*, 1996) also showed that foetal calves had higher than/or comparable levels of CD2+, CD4+ and CD8+ T cells in the spleen, thymus, blood as compared to adult cattle. This may lend further credence to the fact that ruminant foetuses are more mature at birth and may partially explain the comparable PRR expression. This may be in sharp contrast to murine foetuses that are known to have a very under developed immune system at birth compared to humans (Durandy, 2003; Renz and Herz, 2002). Other workers have found comparable levels of PRRs in other foetal tissues and cells compared to adult equivalents. Nishimura and Naito (Nishimura and Naito, 2005) also found comparable levels of TLRs in human foetal and adult liver. Reid and co-workers (Reid *et al.*, 2004) have also shown positive staining for dectin-1 on murine spleen at birth, with the staining pattern resembling adult architecture two weeks after birth. Harju and co-workers (Harju *et al.*, 2001), found that in mice the levels of TLR 2 and 4 increased constantly in the lung from conception through birth until adults. In contrasts, the expression in the liver was the comparable between the foetal, neonate and adult mice. Viemann and co-workers (Viemann *et*

al., 2005) also found comparable levels of TLR4 in human neonates peripheral blood monocytes and adults but found a statistically lower expression of TLR2 in neonates compared to adults.

The lower expression of CD14 in foetal tissues as compared with adult tissues would agree with the findings of Jones and co-workers (Jones *et al.*, 2002) who found a higher expression of CD14 in adult monocytes compared to third trimester foetal cells. As CD14 is GPI anchored and has no intracellular signal transduction but works with TLR4, it was interesting to note that the expression of TLR4 was also significantly higher in adult skin than foetal skin. TLR4 expression was also higher in adult spleen although the difference was not significant. This could show the association of these two PRRs in innate immunity.

Histology of the foetal spleen however may partially explain this – the foetal spleen has early aggregation of lymphoid cell patches distributed throughout. Although reticulocytes make up the predominant cell type, there is evident partial differentiation of white and red pulp and their putative constituent cellular – and this may account for the full profile of PRRs seen. DCs are among the first cells to colonise the spleen during development, thus it would be reasonable to assume that these spleens would have copious quantities of DCs at this stage. This increasing proportion of lymphoid cells in the ovine foetal spleen agrees with that of Al Salami and co-workers (Al Salami *et al.*, 1985) who report that white pulp expands from day 70 of gestation to occupy a predominant histological proportion of splenic architecture at birth. Similarly, foetal skin also seems well-endowed with a rich cellular infiltrate that could possibly be immune cells expressing high levels of PRRs.

Comparative immunostaining of the foetal tissues sections to identify the cell types present would have been of additional value in further characterization of the cell types that may be responsible for the PRR expression and their relative densities in comparison to adult tissues.

One possible explanation for the comparable levels of PRRs in foetal tissues compared to adult tissues (but with an obvious under developed neonatal immune

response) is that the levels are comparable but the PRR activity is hampered by the presence of an inhibitory plasma factor in foetuses as described by Levy and colleagues (Levy *et al.*, 2004). They found inhibitory activity to TLRs in neonatal blood that could possibly explain the lower TNF-α expression in monocytes after LPS stimulation compared to adult monocytes. This inhibitory activity was absent in adult plasma. Unfortunately, this factor has not been identified yet and when it is the determination of the expression levels over the gestation period may be very revealing and provide further insight on neonatal innate immune development.

This study showed that foetal spleen has more PRRs that were comparable to adult tissue than the skin. Deducing from these results in the sheep, it would be plausible that in primary and secondary lymphoid organs foetuses develop comparable levels with adults early during gestation but in other tissues the levels increase slowly as the tissues are seeded with PRR-expressing cells based on need and/or demand. For example Carolyn (Carolyn, 1989) proposed that Langerhans cell density in human foetal skin only increases to comparable adult levels in the third trimester. The third trimester human foetus would developmentally be equivalent to the second trimester sheep foetus, thus one would expect that the second trimester foetus would have a full complement of immune cells in the skin. The pre-third trimester skin has statistically less LC than adult skin and their morphology differs markedly. This may have an important bearing on PRR expression and antigen recognition and presentation. The other possible explanation is one based on the findings of Piotrowski (Piotrowski and Croy, 1996) that maternal cells are distributed in the foetus and the presence of these cells could rationalize the PRR mRNA expression seen. This would however require further investigation.

In conclusion, the present study shows that second trimester ovine foetuses have a significantly developed array of innate immune PRRs despite having generally under-developed immune responses at birth. These results would indicate that the repertoire of PRRs expressed by ovine foetuses would enable the foetus to respond to a large variety of pathogen associated components prior to birth and therefore should be able to elicit immune responses. The reason for the underdeveloped immune responses would thus lie beyond the pattern recognition stage and could possibly be

anywhere from the downstream signalling, antigen presentation or with the effector arm of the immune system. These studies further suggest that the foetal immune system is able to recognize and respond to a number of pathogens such as bacteria, viruses, and fungi via PRRs and alert the adaptive immune system. This would form the beginning of the process of eliminating and/or controlling the offending pathogen. The presence of PRRs in the foetus may take place in the development of and maintenance of foetal/neonatal tolerance since it is now well known that PRRs may be involved in tolerance induction and maintenance.

These results will contribute towards the more thorough understanding of the ontogeny of the immune system in sheep and the role that PRRs play therein. They will also form the basis for future research on the role of PRRs in foetal immunology and study the inducibility of these PRRs in foetal disease conditions. This will increase the understanding of the fundamental processes during immune development *in utero* and how the foetus is able to selectively detect microbial infections whilst not mounting an immune response to the mother. This increased understanding will culminate in our increased ability to treat and prevent neonatal infections.

5 PRR expression in Immune cell sub-sets

5.1 Introduction

Recognition of pathogens by the innate immune system is a decisive step in the initiation of adaptive immune responses. The primary sensors of pathogens and PAMPs are PRRs found on cells of the innate and adaptive immune system, and their activation leads to signalling pathways that culminate in immune and inflammatory gene induction. DCs are a sparsely and widely distributed heterogeneous leucocyte population specialized in the recognition, uptake and processing of antigen for presentation to naïve T cells (Hart, 1997; Mellman and Steinman, 2001). DCs subsets express a wide repertoire of PRRs (Akira *et al.*, 2001; Takeda *et al.*, 2003; Werling *et al.*, 2006) and the ligation of these PRRs leads to DC maturation and migration to secondary lymphoid organs where they are able to effectively prime naïve T cells. It has been proposed that the PRRs engaged on the DC subsets determine the cytokines released (Gautier *et al.*, 2005; Ito *et al.*, 2002) and together these will influence the terminal differentiation of T helper cells into Th1, Th2 or Treg phenotypes (Mazzoni and Segal, 2004). Therefore DCs, by virtue of their sentinel function, are able to amplify the innate immune response and also determine the quality, quantity and functional polarization of the adaptive immune response based on the tissue milieu information gathered via PRRs. Species differences exist in the DC classification criteria and also PRR expression profiles.

Monocytes and macrophages have a critical role in innate immune response and possess the capacity to phagocytose pathogens and particulate antigen and are known to express PRRs. Monocytes/macrophages also utilize PRRs to recognize pathogens and to facilitate uptake.

Ruminant afferent lymph DC subsets (based on CD172a expression) have been shown to preferentially produce cytokines that may polarize T responses. Bovine $CD172a^{+}$ DCs have been shown to preferentially produce IL-10 and bias towards Th2 immune responses while the $CD172a^{-}$ DCs preferentially produce IL-12 and bias towards Th1 immune responses (Stephens *et al.*, 2003). Ovine CD172a subsets have also been shown to produce similar cytokines to the bovine subsets (Matthews *et al.*, 2006). The ovine $CD172a^{+}$ DCs, that also express CD4 and CD8, seem to be broadly

aligned to human and murine plasmacytoid DCs (pDCs) and they express IL-10 but not IL-12 and thus potentially skew immune responses towards Th2 bias. I hypothesized that the two ovine DC subsets ($CD172a^+$ and $CD172a^-$) express different PRRs and are thus capable of responding to different PAMPs (and pathogens). This difference in PRR would thus endow each subset with the unique capacity to prime naïve T cells towards Th1, Th2 or Treg polarization. I further hypothesize that monocytes will express all PRRs to reflect their broad-spectrum role of antigen recognition and pathogen engulfment in innate immunity.

PRRs have been extensively studied in cells of the innate immune system, mainly monocyte/macrophage lineage myeloid APCs. However, PRRs are also expressed on lymphocytes (Zarember and Godowski, 2002). B and T cells are key mediators of mammalian immunity. Priming of naïve T cells by DCs is the first step towards generating an adaptive immune response. It is known that activated APCs are capable of priming T cells and TLR ligation is a primary means by which APC activation occurs (Iwasaki and Medzhitov, 2004). B cells are however able to directly recognize naïve antigen via B cell receptors and are also known to have APC properties (Rodriguez-Pinto, 2005). PRR expression on B cells thus facilitates their APC function but the functional importance of PRR expression on lymphocytes is poorly understood.

The purpose of this study was to determine if ovine T cells and B cells express PRRs as has been described in other mammalian species.

5.2 Flow cytometry – Identification of cell populations

DCs and PBMCs were enriched on density gradients and cell subsets separated by fluorescence activated cell sorting (FACS) (Section 2.2.4.). DCs were identified based on their characteristic high side scatter and forward scatter profiles and electronically gated. These cells were separated into CD172a+ and CD172a- populations by two-colour FACS based on high MHC class II expression and expression or absence of positive staining to CD172a. Gradient separated PBMCs were identified by their characteristic side scatter and forward scatter profiles and further discriminated as B cells, CD4/CD8 T cells and macrophage/monocytes by positive staining with relevant fluorescence labelled monoclonal antibodies (Section 2.2.4.5 and Table 2.1). Expression of cell surface markers was determined by FACS and compared with isotype controls.

Live cells were sorted based on these criteria and the sorted cell populations were determined to be greater than 96% pure by FACS. Analysis and graphing of FACS data was performed using WinMDI software.

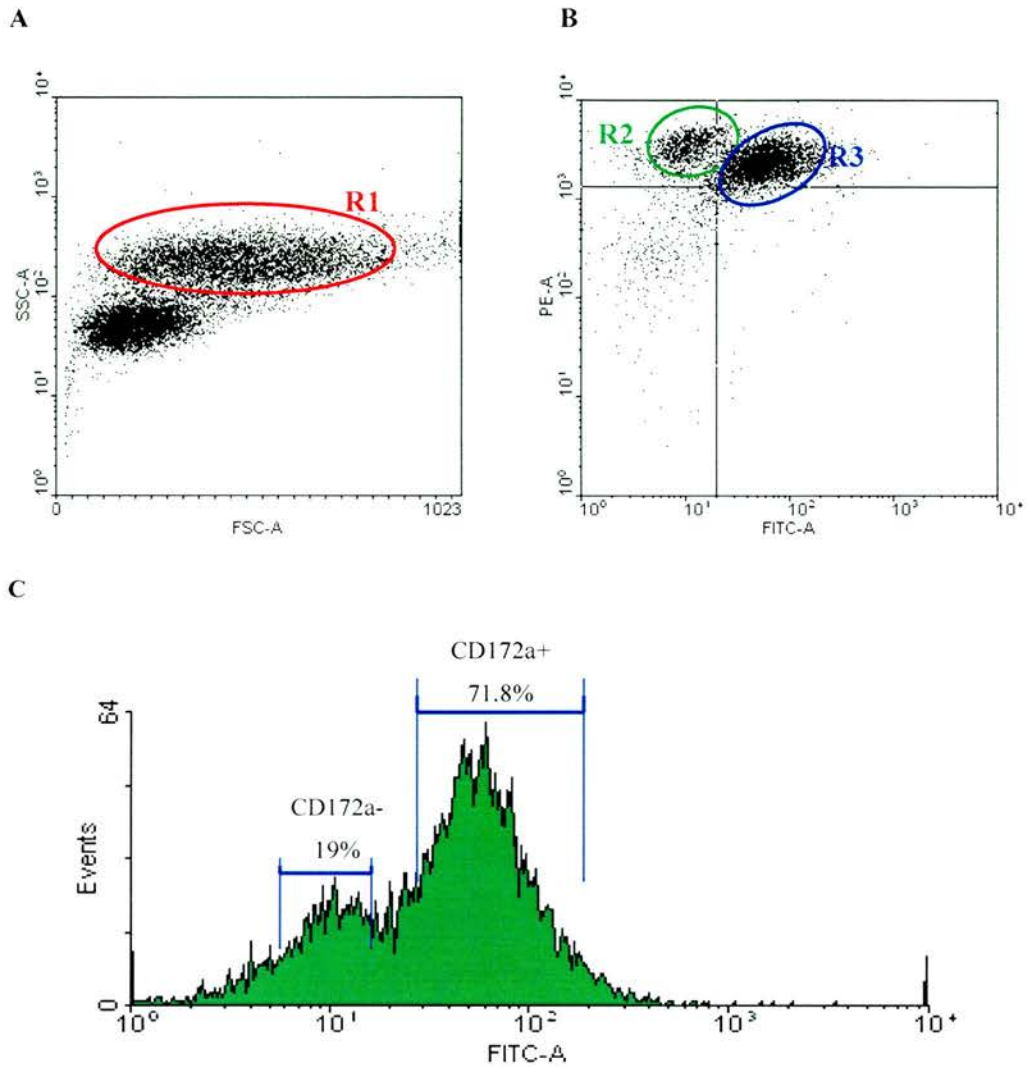
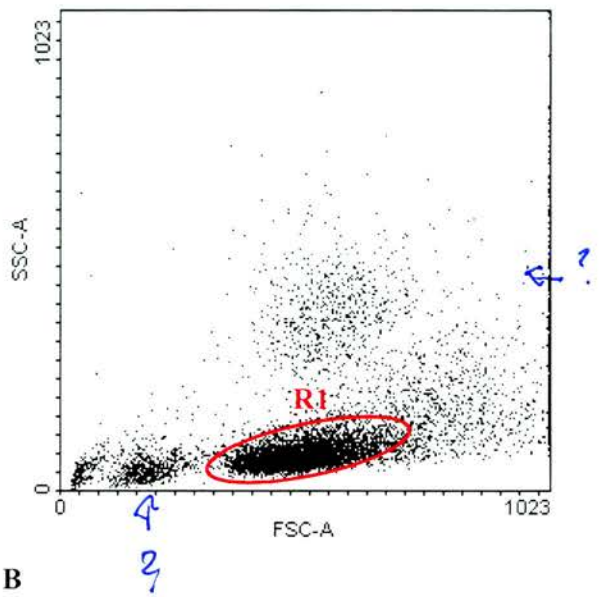


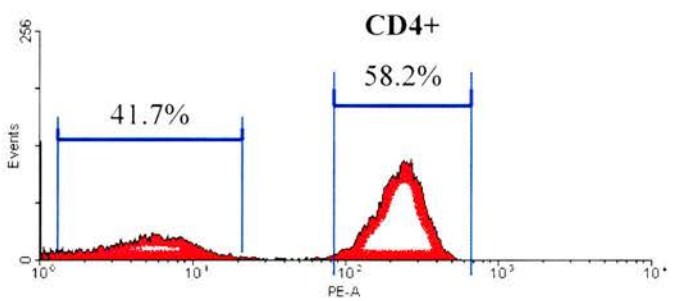
Figure 5.1 FACS of Afferent Lymph Dendritic Cells

Phenotypic characterization and analysis of ALDCs by two colour immunofluorescence flow cytometry. Typical morphology and sorting criteria for DCs **(A)** Forward scatter and side scatter profiles of ALDCs showing the electronic gate (R1) used to isolate DCs. **(B)** Dot plot of R1 gated cell population stained with FITC-conjugated mAb IL-A24 (anti-CD172a) and PE-conjugated SW73.2 (anti-MHC class II) showing the gates for the CD172a- (R2) and CD172a+ (R3) population **(C)** Histogram showing the expression of FITC in the F1 gated cells with two merging peaks of events. ***Abbreviations***: **UL**-upper left, **UR**-upper right, **LL**-lower left, **LR**-lower right

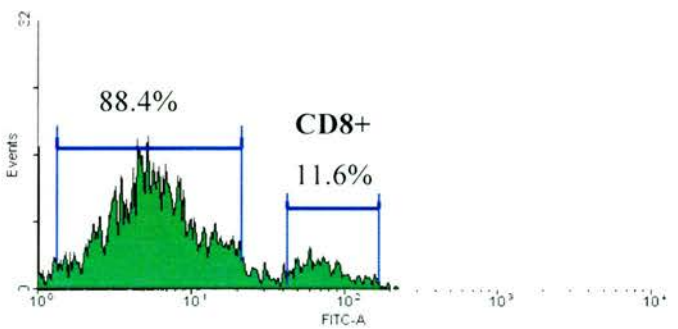
A


1023
SSC-A
0
R1
0
1023
FSC-A

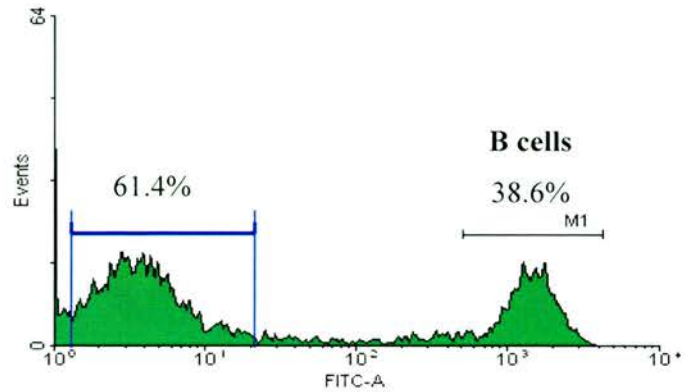
B


CD4+
58.2%
41.7%
256
Events
0
PE-A

C


88.4%
CD8+
11.6%
Events
FITC-A

D



E

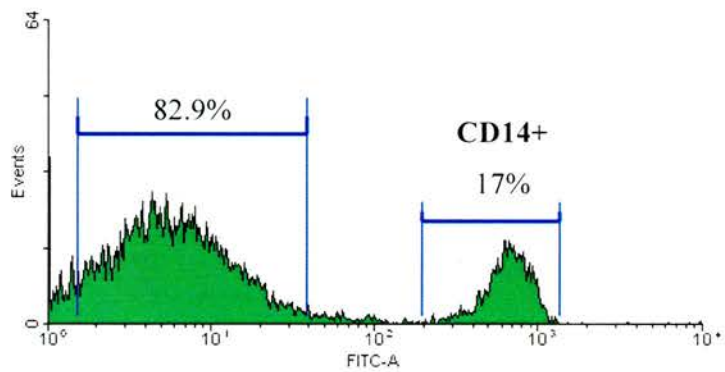


Figure 5.2 FACS of immune cell population defined by the FSC and SSC and stained with the relevant mAb.

Phenotypic characterization and analysis of PBMCs by single colour immunofluorescence flow cytometry. Typical morphology and sorting criteria for PBMCs derived CD4+ cells **(A)** Forward scatter and side scatter profiles of PBMCs showing the position of the electronic gate (R1) used to isolate lymphocytes. **(B)** Histogram of gated CD4 T cell population stained with PE-conjugated biotinylated mAb SBU-T4 showing the significant expression of PE in the gated cells. **(C)** Histogram of gated CD8 T cell population stained with mAb SBU-T8 showing the significant expression of FITC in the gated cells. **(D)** Histogram of gated B cell population stained with mAb VPM30 showing the significant expression of FITC in the gated cells. **(E)** Histogram of gated monocytes stained with mAb VPM65 showing the significant expression of FITC in the gated cells.

***Abbreviations*: UL**-upper left, **UR**-upper right, **LL**-lower left, **LR**-lower right

5.3 PRR expression in different immune subsets

In order to determine the presence of PRR transcripts in different immune system cell subtypes, PRR expression in FACS purified cell subsets of blood PBMCs and lymph was examined using quantitative real time PCR as outlined in Chapter 2.

Due to the low starting amount of RNA, the expression levels of the PRRs was at the lower limit of quantitation for the qPCR assays, thus quantitative analysis could not be carried out. Qualitative analysis of the presence of PRR transcripts was performed using melt curve analysis and gel electrophoresis analysis of qPCR products for the samples and scored. Figure 5.3 shows a representative sample of gels with qPCR amplicons from the cells subsets.

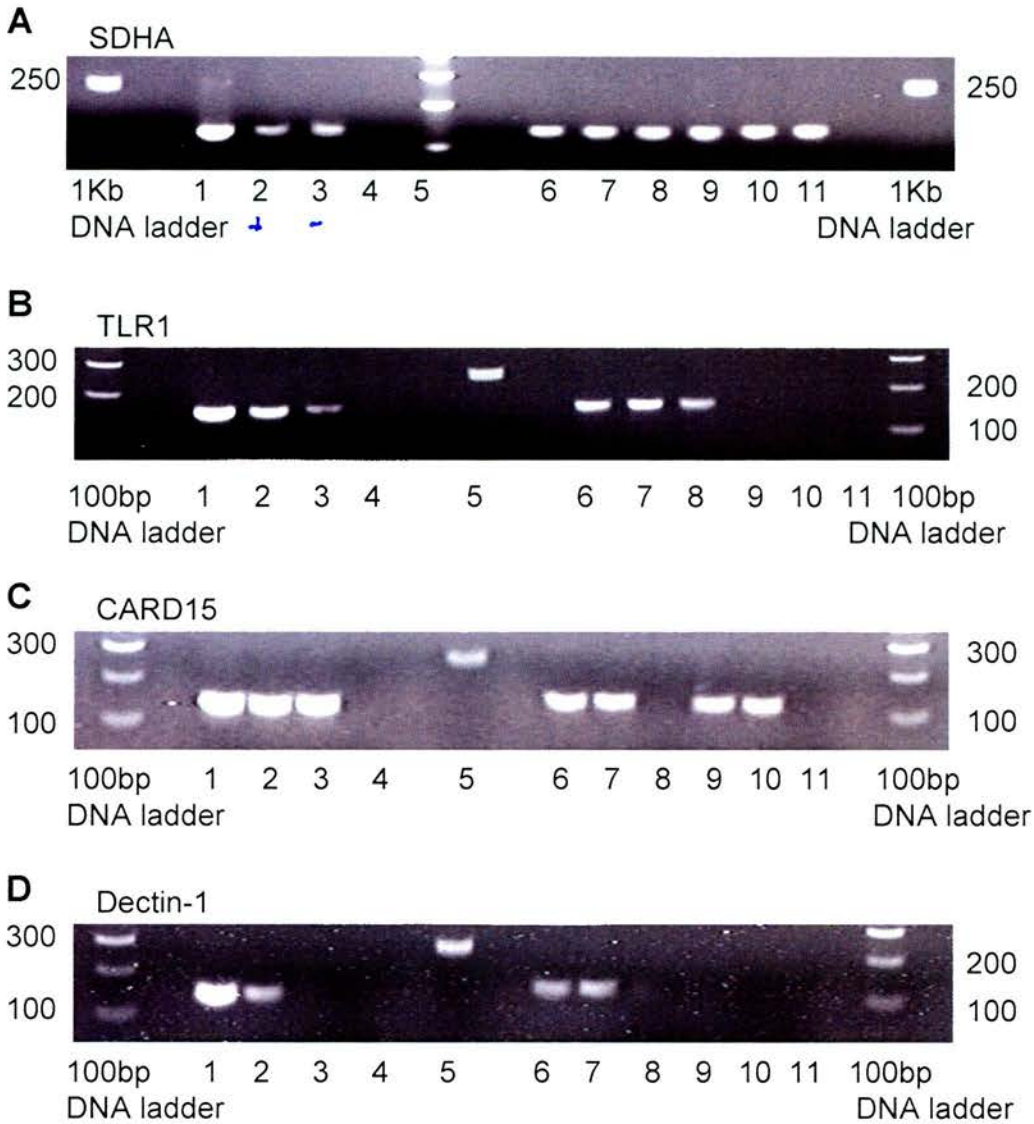


Figure 5.3 Agarose gels showing representative samples of PRR qPCR amplicons

Ethidium bromide agarose gels of representative samples of PRR amplification products from qPCR. Lane 1(plasmid DNA positive control), Lane 2(CD172a+ DCs), Lane3 (CD172a- DCs), Lane 4 (negative control), Lane 5 (DNA ladder), Lane 6 (blood CD14+), Lane 7 (lymph CD14+), Lane 8 (B cells), Lane 9 (Blood CD4+), Lane 10 (Lymph CD4+), Lane 11 (blood CD8+), **A)** SDHA housekeeping gene **B)** TLR1 **C)** CARD15 **D)** Dectin-1.

Table 5.1 PRR expression in different ovine cell sub-sets

	Dendritic Cells		Blood and lymph derived cells					
PRR	**CD172a +ve**	**CD172a -ve**	**Blood CD14**	**Lymph CD14**	**B cells**	**Blood CD4**	**Lymph CD4**	**Blood CD8**
TLR 1	+ve	+/-ve	+ve	+ve	+ve	-ve	-ve	-ve
TLR 2	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
TLR 3	+ve	+/-ve	+ve	+/-ve	-ve	-ve	-ve	-ve
TLR 4	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve
TLR 5	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
TLR 6	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
TLR 7	-ve	-ve	+ve	+/-ve	+ve	+/-ve	-ve	-ve
TLR 8	+ve	-ve	+ve	+ve	+/-ve	+ve	-ve	-ve
TLR 9	+ve	-ve	+/-ve	+/-ve	+/-ve	-ve	-ve	-ve
TLR 10	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
MyD88	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
CARD15	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve
CD14	+ve	+/-ve	+ve	+ve	+/-ve	+/-ve	+/-ve	-ve
Dectin-1	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
Dectin-2	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve

Key

-ve : nothing detected or below limit of detection
\+ : strong expression in all samples
+/- : barely detectable expression or expression in one out of four samples examined

Table 5.2 PRR expression from present study compared with other referenced findings

	Dendritic Cells		Blood and lymph derived cells					
PRR	**CD172a +ve**	**CD172a -ve**	**Blood CD14**	**Lymph CD14**	**B cells**	**Blood CD4**	**Lymph CD4**	**Blood CD8**
TLR 1	1	1	1, 8		1,2,3,4,5,6			
TLR 2	1	1	1,7, 8		2,4			
TLR 3	1		8		1,5			
TLR 4	9	9	1,7,8		2	3,8		
TLR 5		1	1,7,8					
TLR 6	1,9	1,9	1,8		2,4,6	3,4,8		
TLR 7			8		1,2,4			
TLR 8		1	1,7,8			3,4,8		
TLR 9	1							
TLR 10			1,8		1,2,6	3,8		
MyD88			8		8	8		8
CARD15	10	10	11					
CD14								
Dectin-1	12		12					
Dectin-2	13		13					

Colour code (Expression patterns from this study)

No expression from present study and references with similar findings

Strong expression from present study and references with similar findings

Barely detectable expression or expression in one out of four samples examined

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[1] (Werling *et al.*, 2006)
[2] (Bernasconi *et al.*, 2003)
[3] (Ignacio *et al.*, 2005)
[4] (Caramalho *et al.*, 2003)
[5] (Dasari *et al.*, 2005)
[6] (Hornung *et al.*, 2002)
[7] (Kokkinopoulos *et al.*, 2005)
[8] (Zarember and Godowski, 2002)
[9] (Kadowaki *et al.*, 2001)
[10] (Hubert *et al.*, 2006)
[11] (Ogura *et al.*, 2001)
[12] (Willcocks *et al.*, 2006)
[13] (Gavino *et al.*, 2005)

Dendritic cell were defined as cells with high forward scatter and high side scatter and expressing high MHC class II in this study (Figure 5.1A and B). The DCs were further differentiated into two subsets based on expression or absence of CD172a (SIRPα) (Figure 5.1B and C). $CD172a^+$ DCs expressed TLR1, TLR2, TLR3, TLR6, TLR8, TLR9, MyD88, CARD15, CD14, Dectin-1 and Dectin-2. Similar to $CD172a^+$ DC population, the $CD172a^-$ DCs expressed TLR2, TLR6, MyD88 and CARD15 in all samples examined (Table 5.1).

Blood monocytes were separated based on the expression of CD14 and characteristic side scatter and forward scatter profiles as shown in Figure 5.2A and E. They expressed TLR1-TLR8, TLR10, MyD88, CARD15, CD14, CARD15, dectin-1 and dectin-2. Lymph derived CD14 positive cells expressed TLR1, TLR2, TLR4, TLR5, TLR6, TLR8, TLR10, MyD88, CARD15, CD14, dectin-1 and dectin-2.

Blood B lymphocytes were separated based on positive staining with the mAb VPM30 and characteristic side scatter and forward scatter profiles as shown in Figure 5.2. B cells expressed TLR1, TLR2, TLR4, TLR6, TLR7, TLR10 and MyD88 in all samples examined.

Blood CD4+ T lymphocytes were separated based on positive staining with the mAb SBU-T4 and characteristic side scatter and forward scatter profiles as shown in Figure 5.2A and B. Blood derived CD4+ T cells showed the presence of TLR4, TLR6, TLR8, TLR10, MyD88 and CARD15. CD4+ cells derived from lymph showed the presence of TLR4, TLR6, MyD88, CARD15 and CD14.

No PRR transcripts were detected in CD8+ T cells. Transcripts for the adaptor molecule MyD88 were however detected.

5.4 Discussion

The last few decades have seen lymphocytes being progressively subdivided into an increasing number of phenotypically and functionally discrete subsets. With the discovery of PRRs, differential expression of these innate immune receptors in the different subsets has been shown as well as species differences.

This study set out to determine the quantitative expression of PRRs in different immune cell types and the CD172a positive and negative DCs. Quantitative expression of the PRRs could not be carried out due to the resulting low transcript levels for the amount of RNA used, thus qualitative scoring was used to determine the presence or absence of PRR transcripts using melt curves and gel electrophoresis of qPCR products.

In this study the PRR expression of $CD172a^+$ and $CD172a^-$ dendritic cells, blood derived CD4+, CD8+ and CD14+ cells, B cells, lymph CD14+ and CD4+ cells was examined. Analysis of PRR expression from these ovine immune cell subsets indicates that APCs express the widest range of PRRs.

Dendritic Cells

The expression of PRRs in the two ovine DC subsets is as outlined in Table 5.1 which shows that the $CD172a^+$ subset expressed a wider range of PRRs than the $CD172a^-$ subset. Unlike the $CD172a^-$ subset, the $CD172a^+$ expressed the nucleic acid sensing PRRs, TLR3, TLR8, TLR9 which would give this subset the capacity to sense nucleic acids such as viral ds and ss RNA and bacterial CpG DNA from bacteria. This study did not detect any TLR4 and TLR5 transcripts in both DC subsets which is in contrast with other workers (Hubert *et al.*, 2006;Werling *et al.*, 2006) but similar to the findings of Kadowaki and colleagues (Kadowaki *et al.*, 2001). Both subsets did not express TLR4 despite that the $CD172a^+$ expressed CD14 the co-receptor for TLR4 mediated LPS recognition. The $CD172a^+$ population expressed the two c-type lectins, dectin-1 and dectin-2, while the $CD172a^-$ population does not. Gavino and colleagues (Gavino *et al.*, 2005) found expression

of dectin-2 in both myeloid and plasmacytoid DCs with the pDCs having a three fold higher expression than myeloid DCs. This study, however, only found the expression of dectin-2 in CD172a$^+$ DCs. In contrast to Willcocks and colleagues (Willcocks *et al.*, 2006) who found dectin-1 expression in both CD172a$^-$ and CD172a$^+$ DCs, this study only found expression of dectin-1 in CD172a$^+$ afferent lymph DCs (ALDC). However, Willcocks and colleagues (2006), report that although dectin-1 is present in both ALDCs subsets, expression is variable was detected in 14% of samples examined. This variable expression could explain why the dectin-1 was not detected in the CD172a$^-$ DC sub-population in the present study since only four samples were looked at. The findings from the present study on c-type lectin expression would confer the CD172a$^+$ the ability to recognize PAMPs such as beta glucans.

The findings from this study show that the CD172a$^+$ and CD172a$^-$ DC subsets express a wide range of PRR and this may have a bearing on their ability to recognize particular pathogens in a subset specific manner and drive appropriate immune responses. The CD172a$^+$ DC subset had the widest repertoire of PRRs and this may confer this subset with a greater ability to recognize more pathogens modulate more pathogen-specific immune responses compared to the CD172a$^-$ DC subset. Bovine CD172a$^+$ DCs were also shown to have transcripts for a larger number of PRRs than the CD172a$^-$ by Werling and colleagues (2006).

Though the ovine CD172a$^+$ population tend to bias towards exclusive IL-10 expression, Th2 responses and plasmacytoid DC phenotype (Matthews *et al.*, 2006), it has a wider TLR expression profile than has been described for human and mouse pDCs. The PRR expression on CD172a$^+$ DCs, and the predominant cytokine that they produce (IL-10), would seem to be in sharp contrast with the Th type/IL-10/IL-12 paradigm. For example, TLR9 ligation by CpG bacterial DNA is expected to generate protective Th1 type immune responses via IL-12 production, but the CD172a$^+$ DCs that expresses TLR9 does not tend to produce IL-12. Stephens and co-workers (2003) did not find specific pDC equivalents in the bovine ALDCs and also showed that the CD172a$^+$ population, though producing predominantly IL-10, produced low IL-12 later on following stimulation. This has been attributed to the fact that the CD172a$^+$ DCs are a more heterogenous population than the CD172a$^-$

population and may contain cells capable of producing IL-12. In the pig, Jamin and co-workers (Jamin *et al.*, 2006) were able to further separate cells derived from $CD172a^+$ DCs in lymphoid organs into plasmacytoid DCs and conventional DC populations based on co-expression of CD4 and CD11R1 expression respectively. Translated across species, this would imply the presence of plasmacytoid DCs within the ovine $CD172a^+$ population. This may explain the wider PRR expression seen in this population by the present study and that of Werling and colleagues (2006).

CD14 cells - Blood and Lymph

The expression of PRRs in CD14 positive cells (monocytes) as outlined in Table 5.1 shows that these cells express all the PRRs studied. The findings on the expression of TLR1, TLR2, TLR4, TLR5, TLR6, TLR8, TLR9 and TLR10 in CD14+ monocytes agrees with the findings of several workers (Visintin *et al.*, 2001; Werling *et al.*, 2006; Zarember and Godowski, 2002) (see Table 5.2). The expression of the c-type lectins on these cells also concurs with the other previous studies (Gavino *et al.*, 2005; Willcocks *et al.*, 2006). The presence of all the PRRs on these cells would confer them with the ability to recognize most PAMPs that they would encounter. The only differences in PRR expression between the blood and lymph derived CD14+ was the expression of TLR3 and TLR7 where the blood monocytes consistently expressed these two PRRs whilst the expression in afferent CD14+ lymph population was inconsistent. Afferent lymph contains some monocyte/macrophages and some $CD14^{low}$ $CD11^+CD172a^+$ DCs, but the cells in this study were FACS gated to excluded the large granular cells (cells with high forward scatter and side scatter) and thus all the DCs. Thus, the CD14+ cells gated from afferent lymph are of the monocyte/macrophage lineage and have similar PRR expression patterns with the blood monocytes.

Lymphocytes

The expression of PRRs in B cells is summarised in Table 5.1 shows that these cells express most of the PRRs studied. Numerous authors concur that B cells have transcripts for all TLRs, but only TLR1 and TLR6-TLR10 have significant expression (Bourke *et al.*, 2003; Hornung *et al.*, 2002; Zarember and Godowski, 2002). The expression of PRRs in B cells by this study is in agreement with a number of other previous studies (Table 5.2).

Similar to the findings of Willcocks and colleagues (2006), this study did not detect any dectin-1 transcripts in B cells. Gavino and colleagues (2005) found that human dectin-2 was expressed in resting B cells but down-regulated following *in vitro* activation of the B cells. Unlike their finding this study did not detect any dectin-2 on B cells, and this may reflect that the predominant population of the B cells examined in this study were in a state of activation. Bernasconi and colleagues (Bernasconi *et al.*, 2003) also showed differential TLR expression in activated B cells, with memory B cells expressing TLR6, TLR7, TLR9, TLR10 at higher levels than naïve B cells.

The presence of a large number of PRRs in B cells would be consistent with the dual role that B cells play in innate as well as adaptive immunity (McHeyzer-Williams, 2003; Rodriguez-Pinto, 2005).

The expression of PRRs in the blood and lymph derived CD4+ T lymphocytes was similar for all the PRRs detected except that the blood derived CD4+ cells had TLR7 and TLR10 transcripts and none were detected in the lymph derived CD4+ cells (Table 5.1). Most afferent lymph CD4 T cells are activated memory CD4 T cells and express CD45RO, with virtually no naïve T cells (MacKay *et al.*, 1990), while blood CD4+ T cells are predominantly naïve. Differential expression of TLRs with activation status has been shown by Xu and co-workers (Xu *et al.*, 2005) who showed that naïve (CD45RA) CD4+ T cells have a lower cell surface TLR2 expression than activated (CD45RO) T cells in human blood.

In contrast with the findings of the present study, Ignacio and colleagues (2005) found that CD4 and CD8 cells in cats express all TLRs. The levels of expression of TLR1 a TLR6 was however very low in their study. Caramalho and co-workers

(2003) also found strong expression of TLR1, TLR2, TLR6, TLR7 and TLR8 in murine CD4+ cells and differences in TLR expression in these cells based on activation status. Pioneer studies on TLR expression in human T cells also reported the mRNA expression of almost all TLRs (Hornung *et al.*, 2002; Zarember and Godowski, 2002).

The results form this study agree with the finding of Willcocks and co-workers (2006) who did not find expression of dectin-1 in bovine $CD4^+$ T cells and B cells. The lack of expression of dectin-1 in these cells is in disparity with the finding of Taylor and co-workers (Taylor *et al.*, 2002) in mice who report expression on CD4+ splenic T cells. Gavino and colleagues (2005) show that dectin-2 expression in CD4+ T cells is present in activated cells but absent from resting CD4+ T cells and the converse being true for CD8+ T cells and B cells. The lack of dectin-2 on blood CD4+ T cells by the present study could be due to their predominant naive status leading to abrogation of dectin-2 expression. This would however, not explain the lack of dectin-2 in the afferent lymph CD T cells, since these are predominantly activated memory T cells.

CD4 molecule expression has species variations with humans having a wider range of cells expressing the CD4 molecule. In the mouse, expression is almost exclusively restricted to the T cell lineage (including the regulatory T cell subset) but in humans numerous cells including B cells (Moir *et al.*, 1999), CD14+ monocytes/macrophages and CD34+ haematopoetic progenitors (Louache *et al.*, 1994). This could explain some of the species variations in PRR expression observed.

The functional importance of PRR expression in T cells is not well defined. Gelman and co-workers (Gelman *et al.*, 2004) propose that direct ligand interaction with PRRs CD4+ T cells enhances their survival and allows them to respond directly to PAMPs in the absence of APCs. They propose that it may be a mechanism by which CD4+ cells may drive adaptive immune responses when APCs are ineffective at T cell priming due to pathogen virulence or immune evasion factors. Other workers (Xu *et al.*, 2005), propose that TLRs may act as co-stimulatory molecules.

Concluding Comments

Some of the differences seen in the PRR expression in the cell populations may be due to the different activation states. Activation or maturation is known to change the PRR expression profiles of cells (Gavino *et al.*, 2005; Gelman *et al.*, 2004; Kokkinopoulos *et al.*, 2005; Visintin *et al.*, 2001;Xu *et al.*, 2005). Cell preparation techniques, although unavoidable, can activate cells and introduce differences in cell PRR expression patterns.

The absence of signal for some of the PRRs in the cell subsets could be due to the fact that most PRRs are known to be expressed in very low copy numbers. It is known that the efficiency of reverse transcription is dependent on RNA template abundance and is significantly reduced when such transcripts are rare in the total RNA (Karrer *et al.*, 1995). Since I started with a very low amount of total RNA, this could explain the absence of the low copy number PRR gene transcripts. Gene specific primers could have been used for RT to increase the specificity but it has the limitation that it requires a lot of RNA and an RT reaction for each gene and each sample. Multiplex gene specific RT reactions can be done but require time-consuming and rigorous optimization. The use of nested PCR would have probably yielded better quantitative data than using the two-step qPCR that I used in this study.

In order to obtain quantitative relationships of PRR expression between the different cell subsets, this work would need to be performed with a higher amount of RNA that will result in high transcript levels. The use of the more sensitive Taqman probe qPCR would also enhance the detection of low copy number transcripts to facilitate quantitative comparisons of gene expression of the ovine PRRs in these immune cell subsets.

6 Pattern Recognition Receptors in ovine paratuberculosis

6.1 Introduction

Paratuberculosis is a chronic, progressive enteropathy of domestic and wild ruminants, first described in cattle by Johne and Frothingham in 1895, and caused by infection with the intracellular bacterium *Mycobacterium avium paratuberculosis (Map).* It has a worldwide distribution and in the United States alone, it is estimated to cost the dairy industry losses in excess of $200 million annually. Ovine paratuberculosis is present as three clinical forms, namely asymptomatic, paucibacillary and multibacillary. Each of these clinical forms correlates with the degree of immunity that the host has mounted against the mycobacterial challenge (Burrells *et al.*, 1998). There are currently no effective vaccines against *Map*. It has been shown that vaccines should be designed to evoke a more specific and most appropriate immune response rather than the most vigorous in order to be protective (Brown *et al.*, 1998; Estes and Brown, 2002). Understanding the regulation of innate immune mechanisms, at a molecular level during *Map* infections, would allow potential manipulation of these mechanisms during the formulation of optimal *Map* vaccines, and would facilitate the development a vaccine with specific and appropriate cell-mediated immune protective mechanisms. However, the overall pathogenesis of JD is poorly understood, but the persistence of *Map* in the macrophages of JD cases is well documented. Ovine JD pathology and lesion development are very similar to human leprosy (Modlin *et al.*, 1988) and the clinical immunopathology of multibacillary and paucibacillary clinical forms of ovine JD translates to lepromatous and tuberculoid leprosy in humans respectively. Similarity to leprosy makes paratuberculosis an ideal disease condition to study mechanisms by which the innate immune system influences host defence mechanisms and modulates adaptive immunity to determine outcome of mycobacterial infection/disease. The paucibacillary and multibacillary forms of the disease represent typical type 1 and type 2 immune responses respectively. Cases of tuberculoid (paucibacillary) leprosy are characterized by type1 cytokine release and better clinical outcome compared to the lepromatous type, with patients being able to contain infection and limit spread.

The ileal histopathology is also different between the paucibacillary and multibacillary forms of JD, with the multibacillary having more macrophages and eosinophils in the intestinal lesions and the paucibacillary form having a higher lymphocyte infiltrate. PRR engagement in antigen presenting cells is know to skew T cell responses towards Th1 (Schnare *et al.*, 2001), Th2 (Re and Strominger, 2001) or T regulatory responses (Zanin-Zhorov *et al.*, 2006). Different PRRs on APCs are able to recognize PAMPs on *Mycobacteria* (Fortune *et al.*, 2004; Tsuji *et al.*, 2000) and these APCs are capable of initiating and driving polarized responses via CD4+ T cells. PRRs engaged at this point may be a critical point in determining the outcome of *Map* infection. However, continued stimulation of the immune system from long standing infection would inevitably lead to continued PRR activation and this could lead to hyperactive immune reactions and pathology. TLR engagement is also known to increase the differentiation of monocytes into macrophages and DCs (Krutzik *et al.*, 2005). This would thus imply that APCs engaged in the different clinical forms of JD as evidenced by the differences in histopathological presentation would result in different levels of PRRs.

Thus this study approached ovine JD as an ideal disease for studying the role played by PRRs in determination of the polarization of Th1 and Th2 responses in mycobacterial disease, and therefore pathology. The hypothesis is that the PRR profiles of the different forms of JD would explain the resultant T helper type immune response and histopathological pictures. Using the PRR-specific quantitative real time PCR assays developed in Chapter 2, comparative PRR expression was quantified in ileum (the target tissue for *Map* infection) from the three clinical forms of JD and a further comparison made with uninfected control sheep.

The response to infection varies between individuals and it is increasingly becoming evident that some of these differences may be due to the host's genetic composition. Polymorphisms and defects in production of immune molecules, such as PRRs would have significant effects and a host's ability to recognize and respond to pathogens and will consequently have relevance to infectious disease susceptibility and immune disorders (Ting *et al.*, 2006). Amino acid changes due to polymorphisms in the recognition domains of these PRRs (the LRR in TLRs and

NODs) would have a more pronounced effect as this would abrogate effective PAMP recognition. In humans, TLR2 exon 2 and CARD15 exon 11 mutations have been described in association with the mycobacterial diseases tuberculosis (Ben-Ali *et al.*, 2004) and leprosy (Kang and Chae, 2001) that are related to ovine JD and also in Crohn's disease (Ogura *et al.*, 2001) that has a similar clinical presentation to JD (Chiodini, 1989; Greenstein, 2003). Direct sequencing was used to search for these equivalent single nucleotide polymorphisms in ovine TLR2 and CARD15 genes in paratuberculosis cases. The hypothesis is that SNPs in either or both CARD15 exon 11 and TLR2 exon 2 could be linked to the different pathological forms of ovine paratuberculosis. The identification of disease predisposing SNPs would facilitate the development of livestock breeding programmes to eliminate predisposed genotypes.

All mycobacterial species express lipoarabinomannan (LaM), a unique, immunostimulatory, cell wall gylcolipid. LaM is known to bind to leucocytes and modulate immune responses in these leucocytes; it has thus been postulated to be a virulence factor. The interaction of LaM with innate receptors like PRRs could influence the outcome of mycobacterial infections. LaM was used to stimulate skin in order to simulate one aspect of *Map* immune recognition and evaluate the post-LaM ligation innate immune response via differential PRR expression by cutaneous immune cells.

Improved understanding of the role played by PRRs in GIT infections could lead to improved prophylactic and therapeutic interventions. This goal will be achieved by enabling targeted manipulations of innate immune mechanisms early during the antigen recognition stage and as antigen processing and presentation takes place to shape the immune response. Effective prophylactic interventions will dramatically reduce the losses that are currently being experienced by the livestock industry worldwide and result in improved food security. Although its zoonotic potential is still very controversial, *Map* has been implicated as the causal organism or exacerbating factor in human Crohn's disease and JD results may have a bearing on the better understanding of Crohn's disease immunopathogenesis.

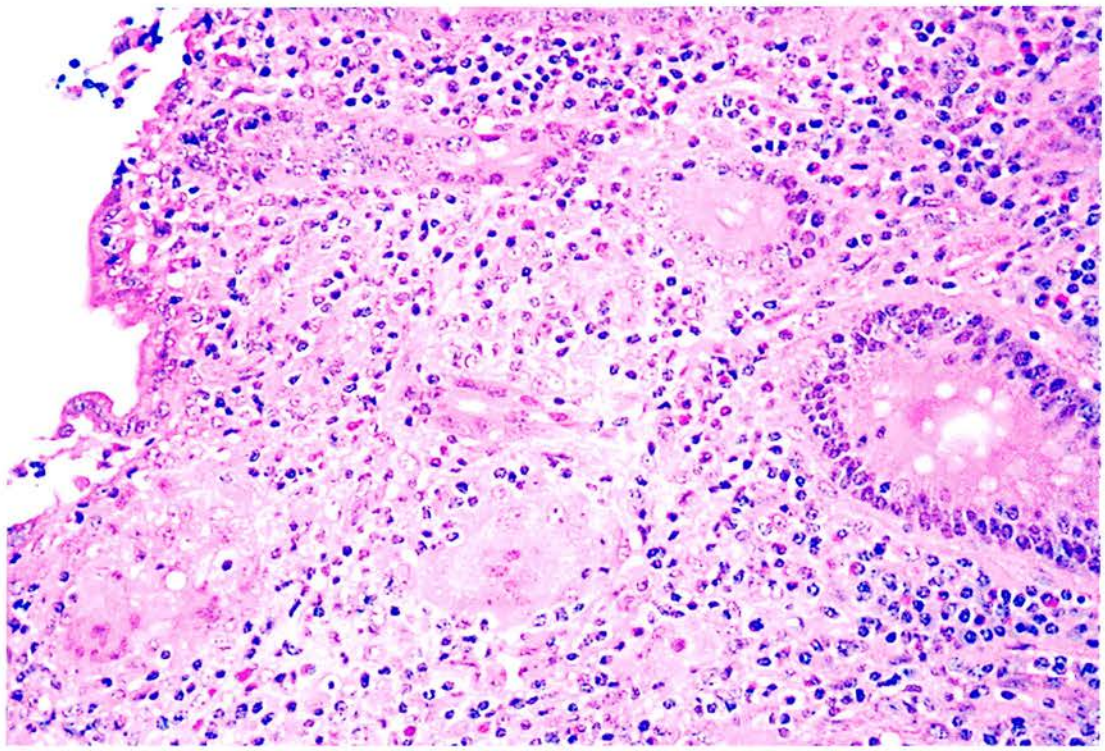
6.2 Results

6.2.1 Definition of ovine JD cases

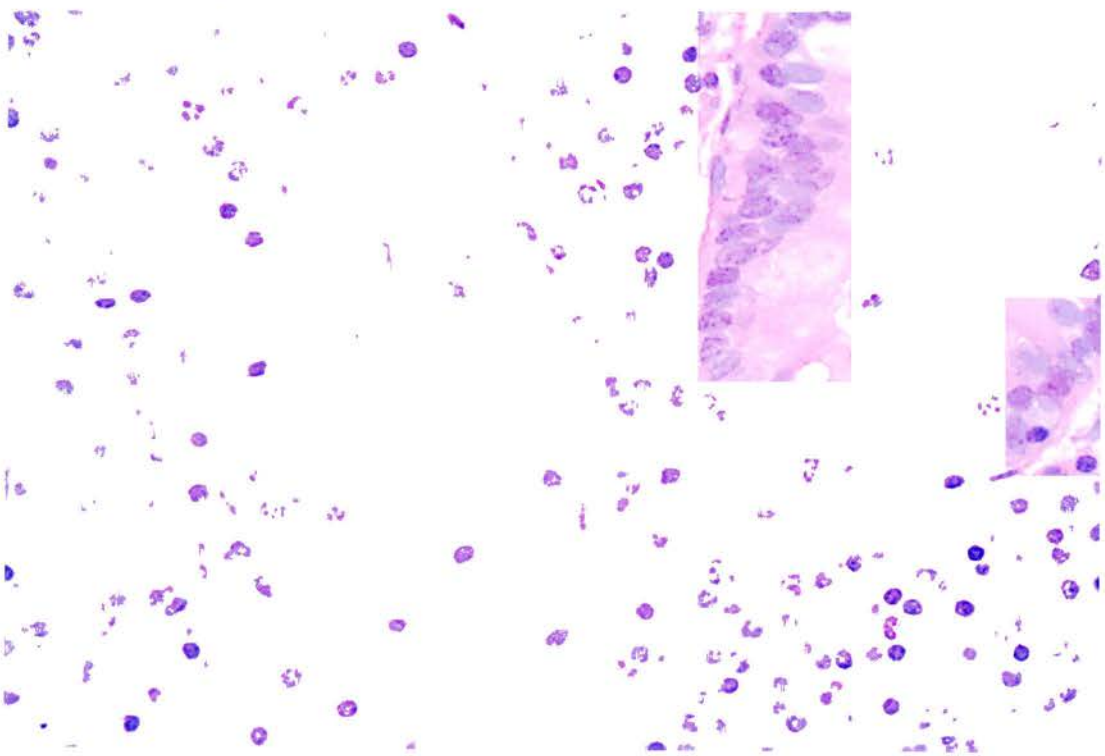
A retrospective study of ovine ileum tissues was carried out from sheep flocks naturally infected with paratuberculosis. Distal ileum samples from 24 cases of Johne's disease diagnosed at post-mortem were utilized. There were 8 cases of each clinical designation and all cases had clinical histories, typical of Johne's disease and all case diagnoses were confirmed by gross pathological changes at necropsy, culture and histopathology at the Moredun Research Institute, Edinburgh. Appendix V has a table summarizing each case signalment.

Figure 6.1A and Figure 6.1B shows haematoxylin and eosin stained paraffin sections of ileum derived from paucibacillary sheep that illustrates the increased lymphocyte infiltration on the lamina propria in this form of ovine JD. Figure 6.1C and Figure 6.1D shows haematoxylin and eosin stained paraffin sections of ileum derived from multibacillary sheep. The sections clearly demonstrate the presence of aggregations of high numbers of macrophages (see black arrows) in the lamina propria of the ileum that are typical of this clinical form of JD. Figure 6.1E shows Ziehl Neelsen (ZN) stained paraffin section of multibacillary JD ileum and clearly demonstrates the red mycobacterial bacilli in the macrophages (see green arrows) against the blue tissue background.

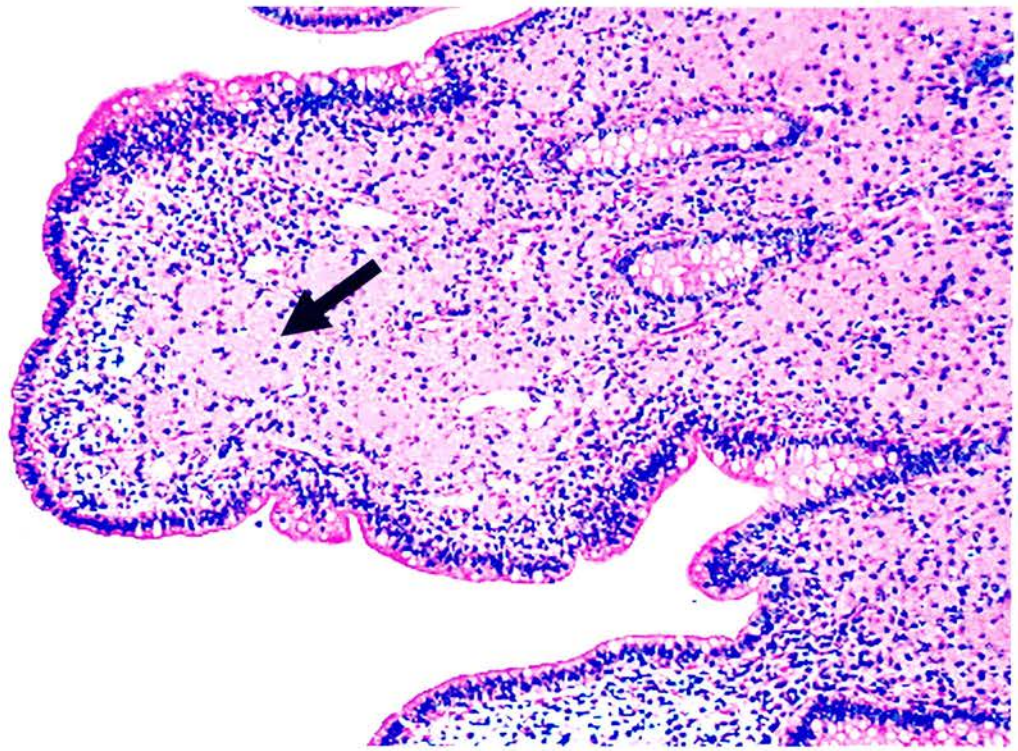
Genomic DNA samples obtained from the multibacillary, paucibacillary and asymptomatic ileum samples were all positive for *Map* IS900 whilst tissues from the control sheep were *Map* negative as determined by the IS900 qPCR assay.



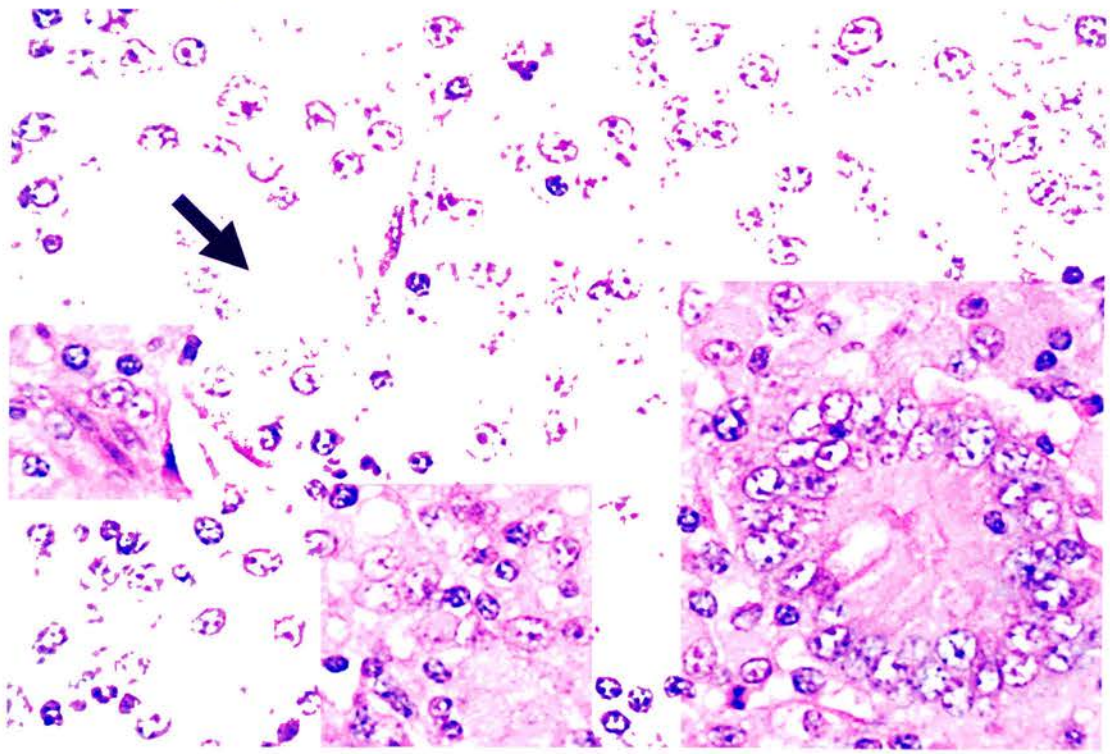
A. Paucibacillary JD ileum x 250



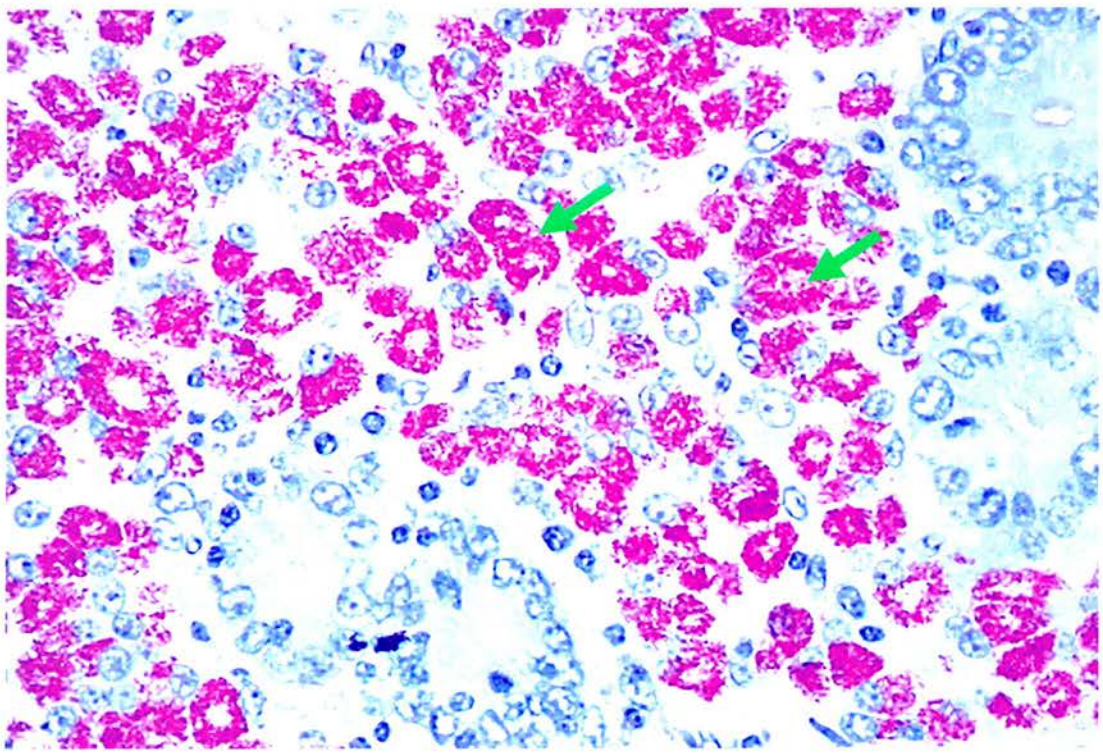
B. Paucibacillary JD ileum x 400



C. Multibacillary JD ileum x 100



D. Multibacillary JD ileum x 400



E. ZN multibacillary JD ileum x 400

Figure 6.1 Histopathological presentation of ileum from the clinical forms of JD

Haematoxylin and eosin stained paraffin section of ileum tissues derived from paucibacillary and multibacillary clinical forms of ovine JD showing the different cellular infiltrate characteristic of each clinical type. **A.** Paucibacillary x 250, **B.** Paucibacillary JD x 400, **C.** Multibacillary JD x 100, **D.** and Multibacillary JD x 400. **E.** ZN stained paraffin section of ileum tissues derived from multibacillary clinical forms of ovine JD. Histopathological sections kindly provided by Dr. Susan Rhind, Veterinary Clinical Studies, University of Edinburgh.

6.3 PRR expression in paratuberculosis ileum tissue

6.3.1 Comparative PRRs expression in ileum derived from the three clinical form of ovine paratuberculosis

In order to evaluate the comparative PRR expression in the three different clinical forms of ovine JD, RT-qPCR was performed using RNA derived from ileum from asymptomatic, paucibacillary and multibacillary JD as outlined in Chapter 2. Figure 6.2 shows the comparative PRR mRNA expression in the three clinical forms. Each graph represents duplicate runs of each sample and shows the individual values (red dots), the mean and 95% confidence interval of the means.

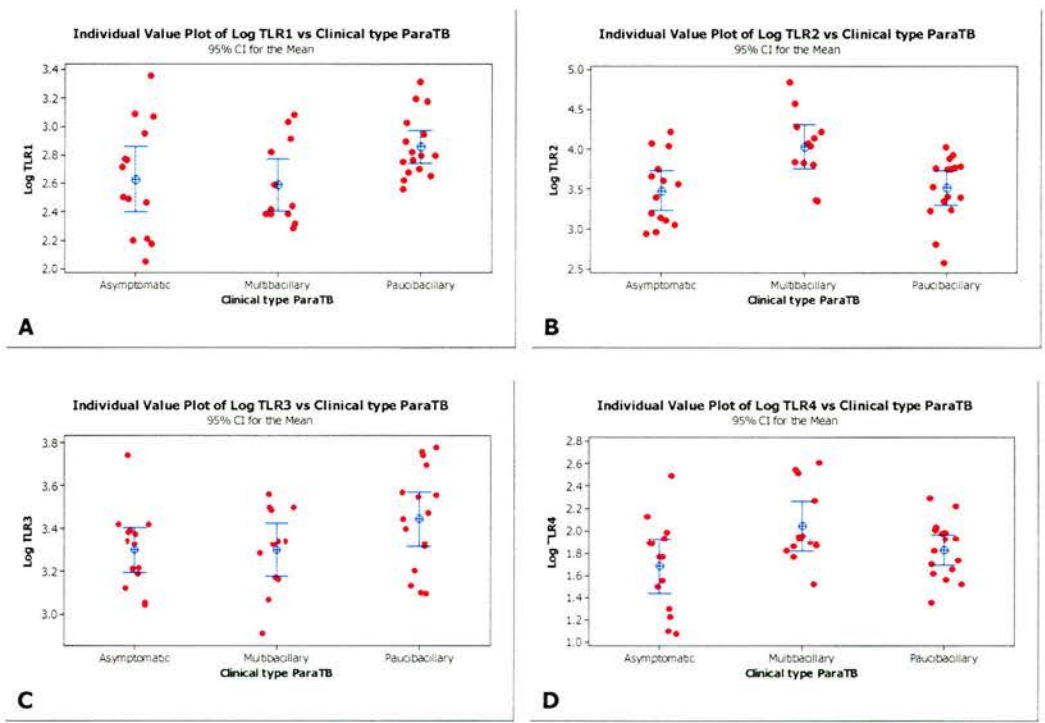


Figure 6.2 Graphical representation of PRR expression in ovine paratuberculosis ileum

RT-qPCR analysis of TLR1(**A**), TLR2(**B**), TLR3(**C**) and TLR4(**D**) transcripts in ileum derived from asymptomatic (n=8), multibacillary (n=6), and paucibacillary (n=8) clinical forms of ovine paratuberculosis. Data are expressed as individual value plots of normalized Log_{10} (copy number per qPCR reaction) of each PRR. The circles on the graphs represent the mean and the whiskers representing 95% confidence interval of the mean.

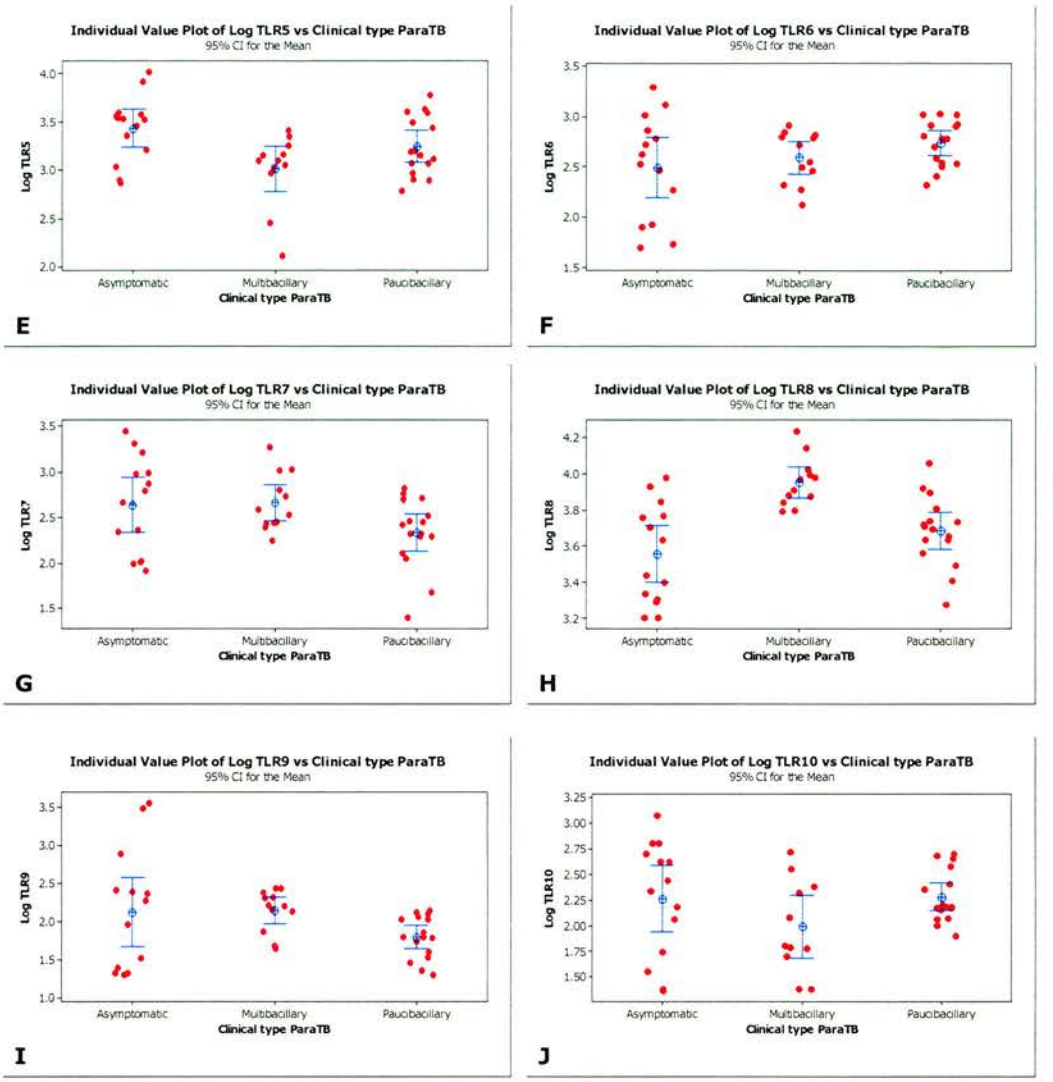


Figure 6.2 Graphical representation of PRR expression in ovine paratuberculosis ileum (continued)

RT-qPCR analysis of TLR5(**E**), TLR6(**F**), TLR7(**G**), TLR8(**H**), TLR9(**I**), and TLR10(**J**) transcripts in ileum derived from asymptomatic (n=8), multibacillary (n=6), and paucibacillary (n=8) clinical forms of ovine paratuberculosis. Data are expressed as individual value plots of normalized Log_{10} (copy number per qPCR reaction) of each PRR. The circles on the graphs represent the mean and the whiskers representing 95% confidence interval of the mean.

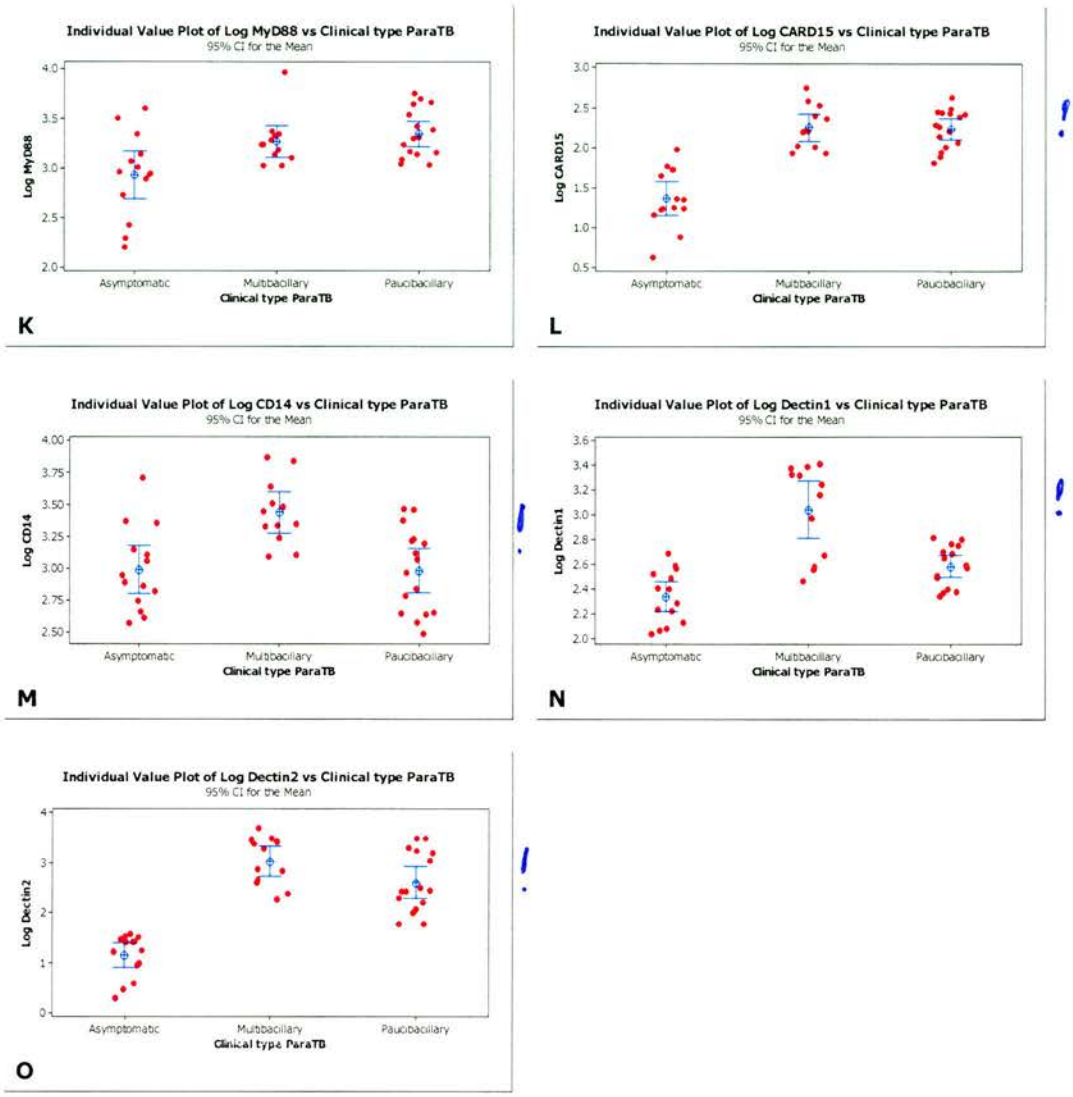


Figure 6.2 Graphical representation of PRR expression in ovine paratuberculosis ileum (continued)

RT-qPCR analysis of MyD88(**K**), CARD15(**L**), CD14(**M**), Dectin-1(**N**) and Dectin-2(**O**) transcripts in ileum derived from asymptomatic (n=8), multibacillary (n=6), and paucibacillary (n=8) clinical forms of ovine paratuberculosis. Data are expressed as individual value plots of normalized Log_{10} (copy number per qPCR reaction) of each PRR. The circles on the graphs represent the mean and the whiskers representing 95% confidence interval of the mean.

Normalized PRR	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8
Paucibacillary	1.31	0.93	1.46	0.95	0.63	1.13	0.36	1.22
Multibacillary	0.78	3.56	1.00	1.81	0.37	0.83	0.75	2.17
Asymptomatic	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Normalized PRR	TLR9	TLR10	MyD88	CARD15	CD14	Dectin1	Dectin2
Paucibacillary	0.12	0.67	2.09	6.36	0.94	1.68	45.73
Multibacillary	0.27	0.43	1.84	6.94	2.43	5.95	87.11
Asymptomatic	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Legend

↓ Expression greater than 2 fold lower

↓ Expression less than 2 fold lower

↑ Expression greater than 2 fold higher

↑ Expression less than 2 fold higher

Table 6.1 Summary of comparative means of PRRs expression normalized to asymptomatic ileum PRR expression.

Table 6.1 summarizes the comparative means of the linear values of PRR expression in the three clinical forms of JD. The means have been tabulated normalized to the mean expression of PRRs in asymptomatic JD ileum (asymptomatic JD given an arbitrary value of 1). The relative mean PRR expressions have been colour-coded according to the fold-change differences as outlined in the legend.

6.3.2 Comparative PRRs mRNA expression in normal ileum, vs. asymptomatic and multibacillary forms of paratuberculosis ileum

Asymptomatic JD does not represent a normal immunological state of the ileum, as although it does not show gross or histopathological lesions, it does have *Map* infection present. In order to establish if differences in PRR expression between asymptomatic JD and normal, uninfected ileum exist, a comparative analysis of PRR expression was undertaken between IS900 negative control ileum tissues and asymptomatic and multibacillary JD ileum. Figure 6.3 shows the comparative PRR expression of the normal control ileum compared to asymptomatic and multibacillary JD ileum tissues.

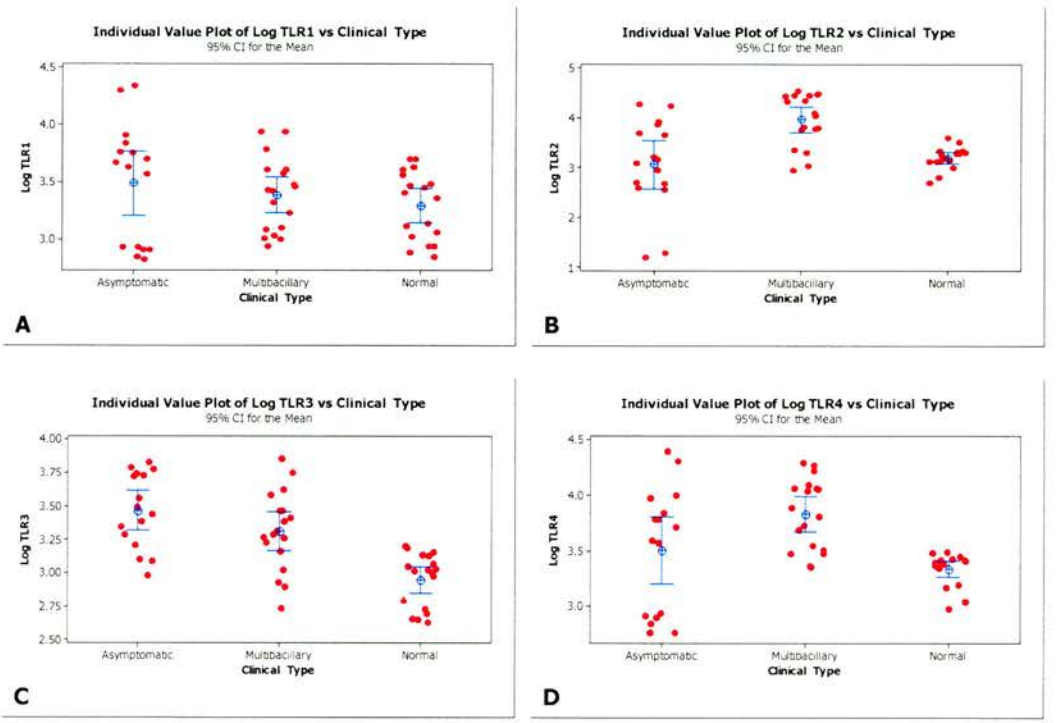


Figure 6.3 Graphical representation of PRR expression in normal ovine ileum compared to paratuberculosis ileum

RT-qPCR analysis of TLR1(**A**), TLR2(**B**), TLR3(**C**), and TLR4(**D**) transcripts in ileum derived from control sheep (n=8) compared to asymptomatic (n=8) and multibacillary (n=8) clinical forms of JD. Data are expressed as individual value plots of normalized Log_{10} (copy number per qPCR reaction) of each PRR. The circles on the graphs represent the mean and the whiskers representing 95% confidence interval of the mean.

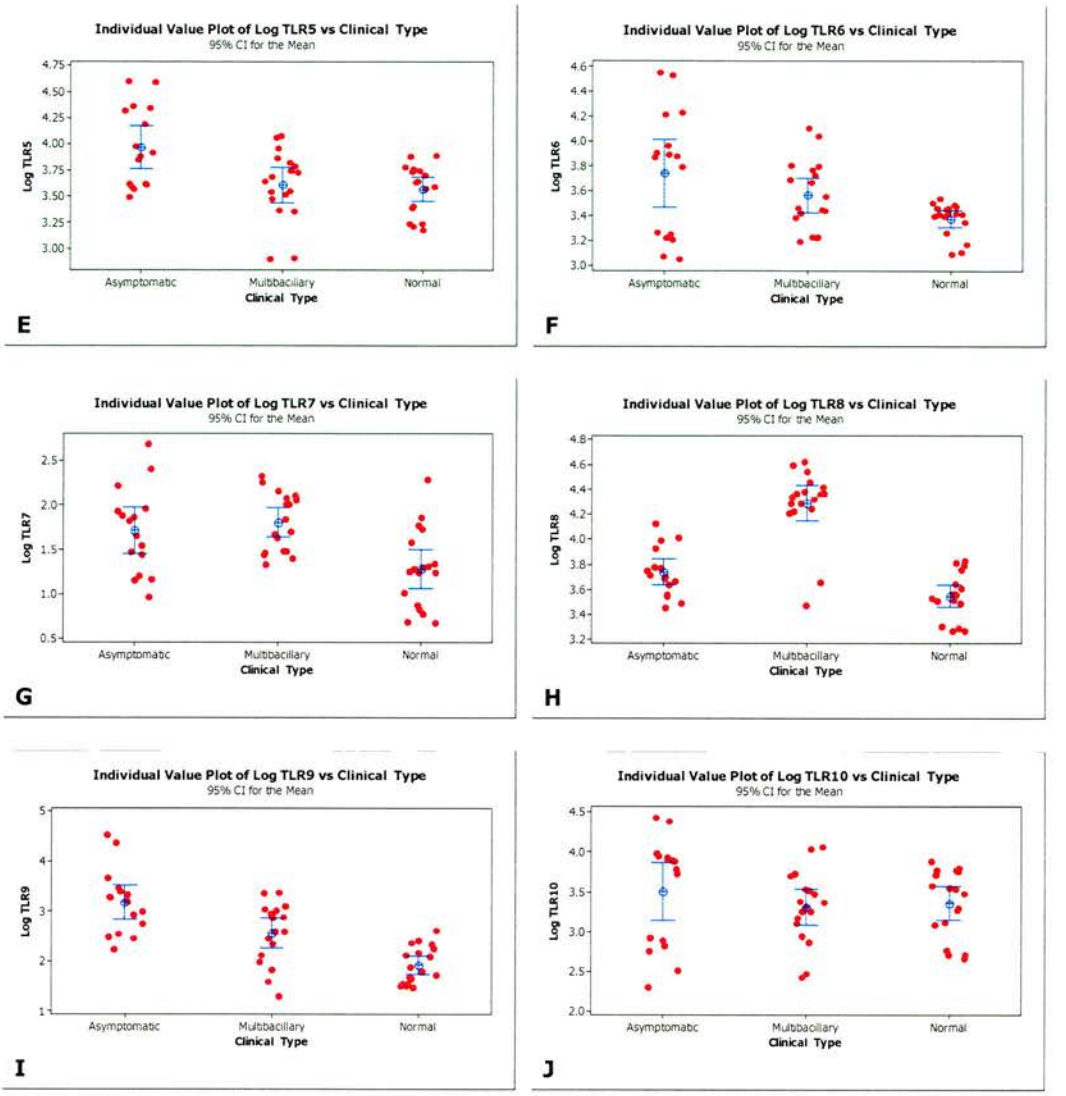


Figure 6.3 Graphical representation of PRR expression in normal ovine ileum compared to paratuberculosis ileum (continued)

RT-qPCR analysis of TLR5(**E**), TLR6(**F**), TLR7(**G**), TLR8(**H**), TLR9(**I**) and TLR10(**J**) transcripts in ileum tissue derived from control sheep (n=8) compared to asymptomatic (n=8) and multibacillary (n=8), clinical forms of JD. Data are expressed as individual value plots of normalized Log_{10} (copy number per qPCR reaction) of each PRR. The circles on the graphs represent the mean and the whiskers representing 95% confidence interval of the mean.

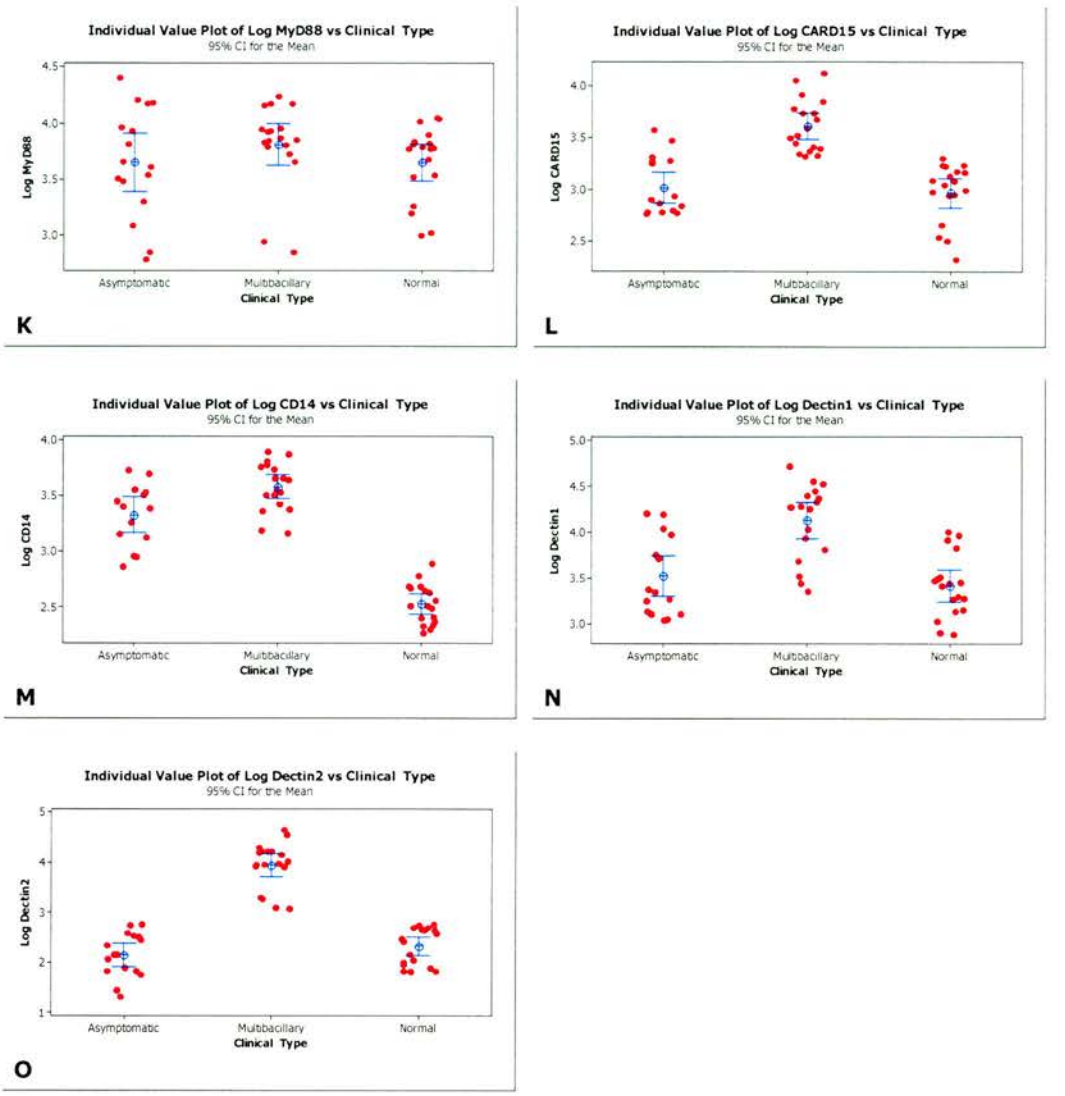


Figure 6.3 Graphical representation of PRR expression in normal ovine ileum compared to paratuberculosis ileum (continued)

RT-qPCR analysis of MyD88(**K**), CARD15(**L**), CD14(**M**), dectin-1(**N**) and dectin-2(**O**) transcripts in ileum tissue derived from control sheep (n=8) compared to asymptomatic (n=8) and multibacillary (n=8), clinical forms of JD. Data are expressed as individual value plots of normalized Log_{10} (copy number per qPCR reaction) of each PRR. The circles on the graphs represent the mean and the whiskers representing 95% confidence interval of the mean.

Mean PRR expression normalized to normal ileum

	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8
Normal	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Multibacillary	1.31	8.68	2.62	3.79	1.22	1.81	2.50	5.89
Asymptomatic	2.35	2.40	3.62	2.79	3.18	3.98	2.83	1.58

Mean PRR expression normalized to normal ileum

	TLR9	TLR10	MyD88	CARD15	CD14	Dectin-1	Dectin2
Normal	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Multibacillary	6.00	0.98	1.47	4.45	12.95	5.31	46.89
Asymptomatic	40.89	2.24	1.31	0.98	6.05	1.48	0.75

Legend

↓ Expression greater than 2 fold lower

↓ Expression less than 2 fold lower

↑ Expression greater than 2 fold higher

↑ Expression less than 2 fold higher

Table 6.2 Summary of comparative means of PRRs mRNA expression normalized to normal ileum PRR expression

Table 6.2 summarizes the comparative means of the linear values of PRR expression in control ileum and the asymptomatic and multibacillary clinical forms of JD. The means have been tabulated normalized to the mean expression of PRRs in the control ileum (given an arbitrary value of 1). The relative mean PRR expressions have been colour-coded according to the fold-change differences as outlined in the legend.

TLR1 expression in ovine JD

Differences in TLR1 expression are not significantly different between the three clinical forms of JD (Figure 6.2A). Multibacillary JD had slightly less TLR1 transcripts than the asymptomatic JD and the paucibacillary JD has 1.3 times more TLR1 mRNA expression than the asymptomatic from. There were also no significant differences in TLR1 expression observed between the control ileum and the asymptomatic and multibacillary clinical forms although they had a higher mean expression of TLR1 than control ileum (Figure 6.3A).

TLR2 expression in ovine JD

Paucibacillary JD had comparable levels of TLR2 mRNA expression with the asymptomatic form of JD (Figure 6.2B) whereas the multibacillary form has greater than three fold more TLR2 than both of the other forms. The difference in TLR2 expression is significantly different between the multibacillary form and the asymptomatic but there is no significant difference between the pauci- and the multibacillary form of JD. Asymptomatic JD had a greater than two fold higher expression of TLR2 than control ileum but this difference was not statistically significant (Figure 6.3B). Multibacillary JD had a greater than eight fold higher expression of TLR2 than the control ileum tissue and the expression was significantly different from both the control ileum and asymptomatic JD.

TLR3and TLR4 expression in ovine JD

TLR3 expression between the three clinical forms of JD is not significantly different (Figure 6.2C). Multibacillary and asymptomatic form had a higher expression of TLR3 than the control ileum but there was no significant difference in TLR3 expression between the control ileum and these two forms (Figure 6.3C).

Multibacillary JD has an approximately 1.8 fold higher TLR4 expression than both asymptomatic and paucibacillary forms (Figure 6.2D). However, the differences in TLR4 expression are not statistically significant. Asymptomatic ileum had a higher expression of TLR4 than control ileum, but the differences were not significant

(Figure 6.3D). Multibacillary JD however, had a significantly higher expression of TLR4 than control ileum.

TLR5 expression in ovine JD

TLR5 expression in asymptomatic sheep is higher than both multibacillary and paucibacillary JD (Figure 6.2E). The multibacillary form has a greater than 2 fold lower mean TLR5 expression than the asymptomatic form of JD and the paucibacillary form has a greater than 1.5 fold lower mean TLR5 expression than the asymptomatic form. The differences in mean TLR5 expression between the three clinical forms are however, not significant. Asymptomatic JD had a higher expression of TLR5 than both control ileum and multibacillary JD (Figure 6.3E). There was no significant difference between the normal control ileum and the multibacillary JD, but the differences between normal and asymptomatic JD were statistically significantly different.

TLR6 and TLR7 expression in ovine JD

TLR6 expression in the three clinical forms of JD had very minor differences that are not significant (Figure 6.2F). Asymptomatic JD ileum had an almost four fold higher expression of TLR6 than normal control ileum and a two-fold higher expression than multibacillary JD. There was however, no significant difference in TLR6 expression between the control ileum and these clinical forms (Figure 6.3F).

Similarly mean TLR7 expression levels between the three clinical forms were comparable with no significant differences (Figure 6.2G). The asymptomatic form of JD had a higher mean TLR7 expression than both multibacillary and paucibacillary forms, being almost three fold higher than the multibacillary and about 1.3 fold higher than the paucibacillary from. Asymptomatic and multibacillary JD had a higher expression of TLR7 than control ileum (Figure 6.3G). Multibacillary JD had a significantly higher TLR7 expression than control ileum. The difference between the control ileum and the asymptomatic JD were not significant.

TLR8 expression in ovine JD

TLR8 transcripts level in the multibacillary form was significantly higher ($p>0.05$) than the other two forms (Figure 6.2H). The multibacillary form had a greater than two fold higher expression of TLR8 than both asymptomatic and paucibacillary forms of JD. The mean TLR8 expression was comparable between the asymptomatic and paucibacillary forms of JD. There was no significant difference in TLR8 expression between the control ileum and the asymptomatic JD (Figure 6.3H). Multibacillary JD had an almost six fold higher expression of TLR8 than the control ileum and the difference was statistically significant.

TLR9 expression in ovine JD

Asymptomatic JD had a higher expression of TLR9 than both pauci- and multibacillary forms (*ca* 8 fold and 3 fold respectively) although there was no significant difference in transcripts between the three clinical forms (Figure 6.2I). Asymptomatic and multibacillary JD both had a higher expression of TLR9 (Figure 6.3I) than control ileum. The difference was statistically significant with the control ileum having a six fold and forty fold lower TLR9 expression compared to the multibacillary and asymptomatic JD respectively.

TLR10 expression in ovine JD

Asymptomatic JD also had a slightly higher mean expression of TLR10 than both paucibacillary and multibacillary JD (*ca* 1.5 fold and 2 fold respectively). These differences were however, not significant (Figure 6.2J). TLR10 expression levels of control ileum were comparable to asymptomatic and multibacillary JD (Figure 6.3J).

MyD88 expression in ovine JD

Pauci- and multibacillary JD had a slightly higher mean expression of MyD88 than asymptomatic JD (*ca* 2 fold and 1.8 fold respectively). The paucibacillary MyD88 expression was significantly higher than the asymptomatic JD. The differences in MyD88 expression between the multibacillary form and the other forms was however, not significant (Figure 6.2K). The clinical forms of JD had slightly higher

MyD88 transcripts level than control ileum but this difference was not significant (Figure 6.3K).

CARD15 expression in ovine JD

Paucibacillary and multibacillary JD had a significantly higher expression of CARD15 than asymptomatic JD (greater than six fold). There was however, no significant difference between the paucibacillary and the multibacillary forms ($p>0.05$) (Figure 6.2L). Asymptomatic JD has comparable expression levels of CARD15 with control ileum (Figure 6.3L). Multibacillary JD however, has a significantly higher expression of CARD15 than both control and asymptomatic JD.

CD14 expression in ovine JD

CD14 expression levels between the paucibacillary form of JD and the asymptomatic were comparable (Figure 6.2M). Multibacillary JD had a higher expression of CD14 than both asymptomatic and paucibacillary forms (greater than two fold). The CD14 expression was significantly different between the multibacillary and the asymptomatic JD but not statistically different between the multibacillary and paucibacillary forms of JD. Multibacillary and asymptomatic JD had a significantly higher expression of CD14 than control ileum (Figure 6.3M). Asymptomatic JD and multibacillary JD have a six fold and 12 fold greater mean expression of CD14 than the control ileum and these differences are statistically significant between all groups.

Dectin-1 and Dectin-2 expression in ovine JD

Asymptomatic JD had a significantly lower mean expression of dectin-1 than both pauci- and multibacillary forms (*ca* 6 fold and 1.5 fold less respectively) (Figure 6.2N). There were also statistically significant differences in the mean expression levels of dectin-1 between the pauci- and multibacillary forms of JD. Similar to dectin-1, the asymptomatic form of JD had significantly lower dectin-2 transcripts than both pauci- and multibacillary forms of JD (*ca* 87 fold and 46 fold less respectively) (Figure 6.2O). There was however, no significant difference in the expression levels of dectin-2 between the multibacillary and paucibacillary forms.

Control ileum has comparable expression levels of dectin-1 with asymptomatic JD (Figure 6.3N). Multibacillary JD however, has a five fold higher mean expression of dectin-1 than control ileum and the difference is statistically different. Control ileum similarly, has comparable expression levels of dectin-2 with asymptomatic JD (Figure 6.3O). Multibacillary JD however, has a greater than forty six fold higher mean expression of dectin-2 than control ileum and the difference is statistically different.

6.4 Lipoarabinomanann (LaM) stimulated skin

6.4.1 Preliminary LaM dosage test experiment

LaM, a major antigenic component of the *Map* cell wall, was to be used as a ligand to stimulate skin in order to evaluate cutaneous immune responses to this important mycobacterial component. In order to determine the dose of LaM to use, three concentrations of LaM were evaluated. Intradermal injections of 100µl of 100µg/ml, 200µg/ml and 400µg/ml LaM in sPBS were tested in a single test animal. As a control, 100µl of sPBS were similarly injected. Skin biopsies were collected as outlined in the materials and methods (Section 2.2.6) and gene expression evaluated for selected genes using qPCR.

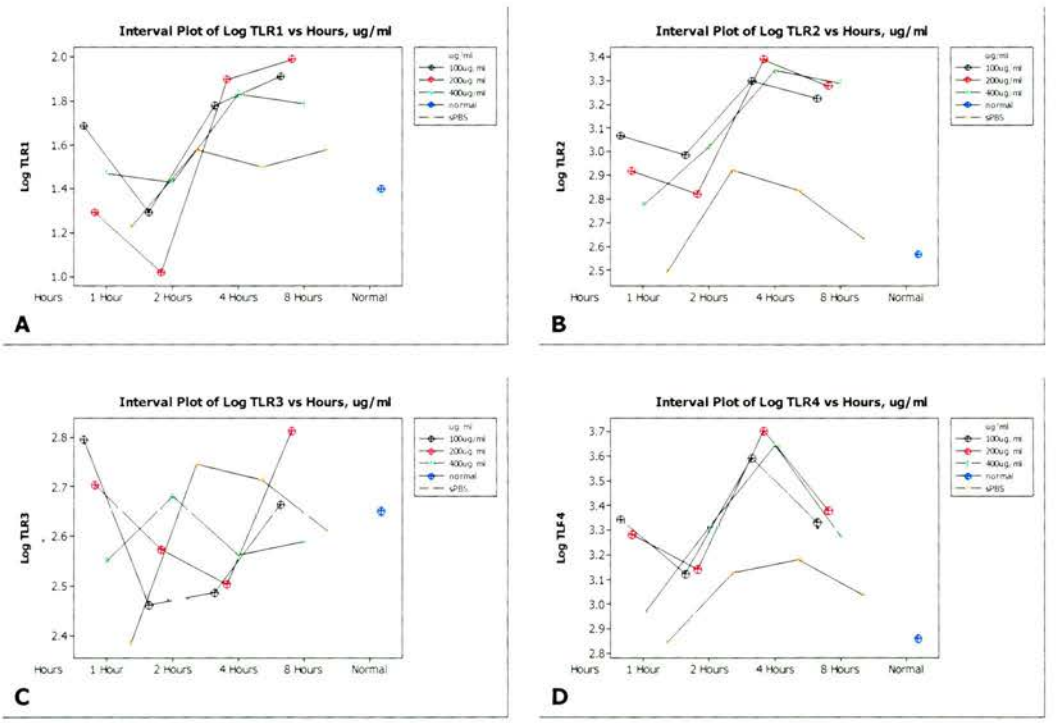


Figure 6.4 Graphical representation of selected PRRs expression in skin stimulated with different doses of LaM

RT-qPCR analysis of TLR1(**A**), TLR2(**B**), TLR3(**C**) and TLR4(**D**) mRNA expression levels in skin biopsy samples treated by intradermal injections of 100µl of 100µg/ml, 200µg/ml and 400µg/ml LaM in sPBS. Data are expressed as mean values of duplicate samples normalized Log_{10} (copy number per qPCR reaction) of each PRR. 100µl of sPBS was injected over the same time periods as a control. The colour-coded circles on the graphs represent the mean for each treatment shown in the legend. Normal represents the mean mRNA expression from untreated skin.

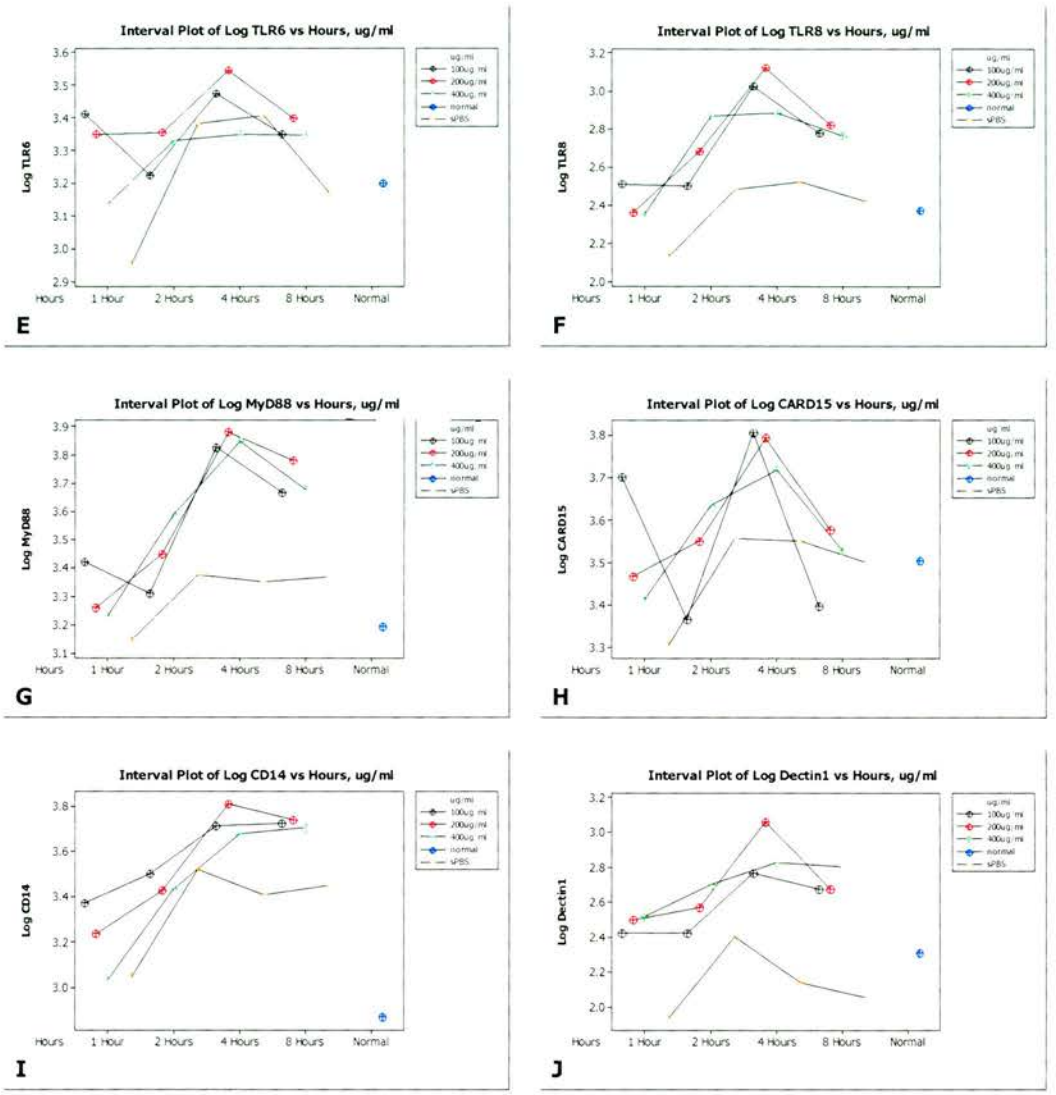
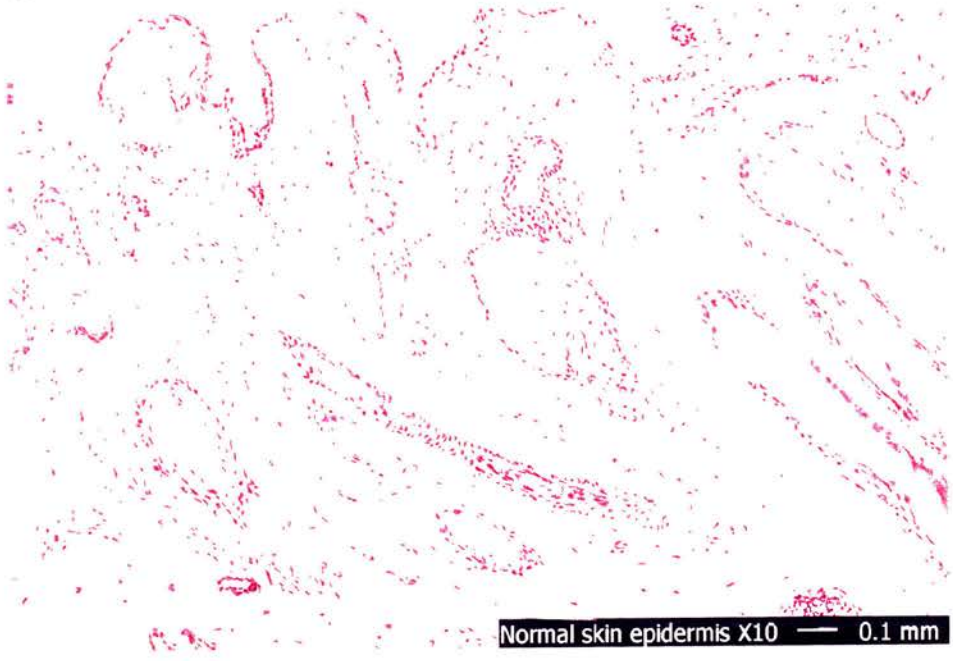


Figure 6.4 Graphical representation of selected PRRs expression in skin stimulated with different doses of LaM

RT-qPCR analysis of TLR6(**E**), TLR8(**F**), MyD88(**G**), CARD15(**H**), CD14(**I**) and dectin-1(**J**) mRNA expression levels in skin biopsy samples treated by intradermal injections of 100µl of 100µg/ml, 200µg/ml and 400µg/ml LaM in sPBS. Data are expressed as mean values of duplicate samples normalized Log_{10} (copy number per qPCR reaction) of each PRR. 100µl of sPBS was injected over the same time periods as a control. The colour-coded circles on the graphs represent the mean for each treatment shown in the legend. Normal represents the mean mRNA expression from untreated skin.

48 max ?

A



B

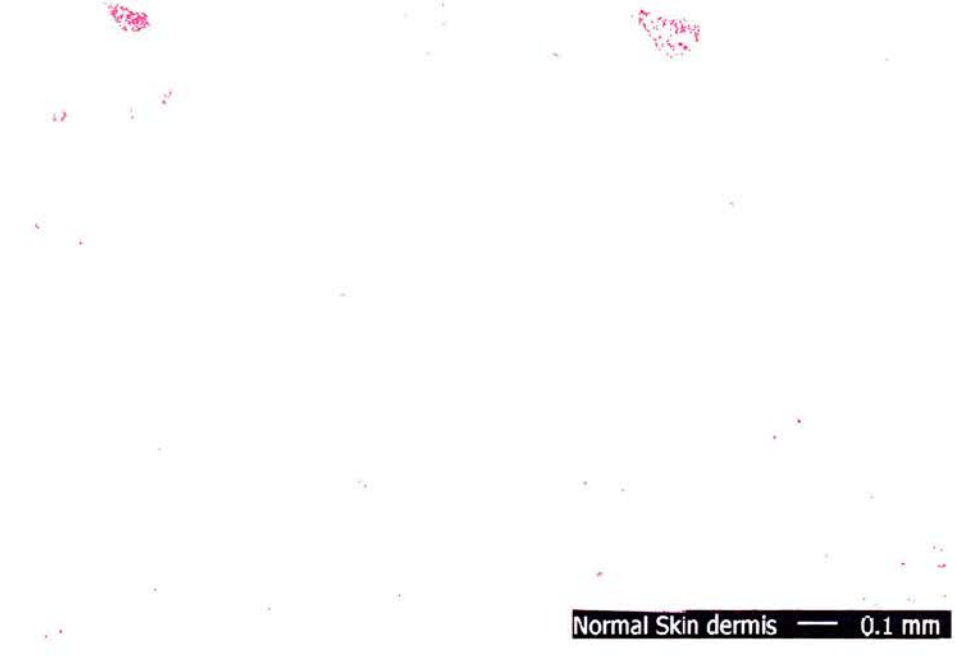
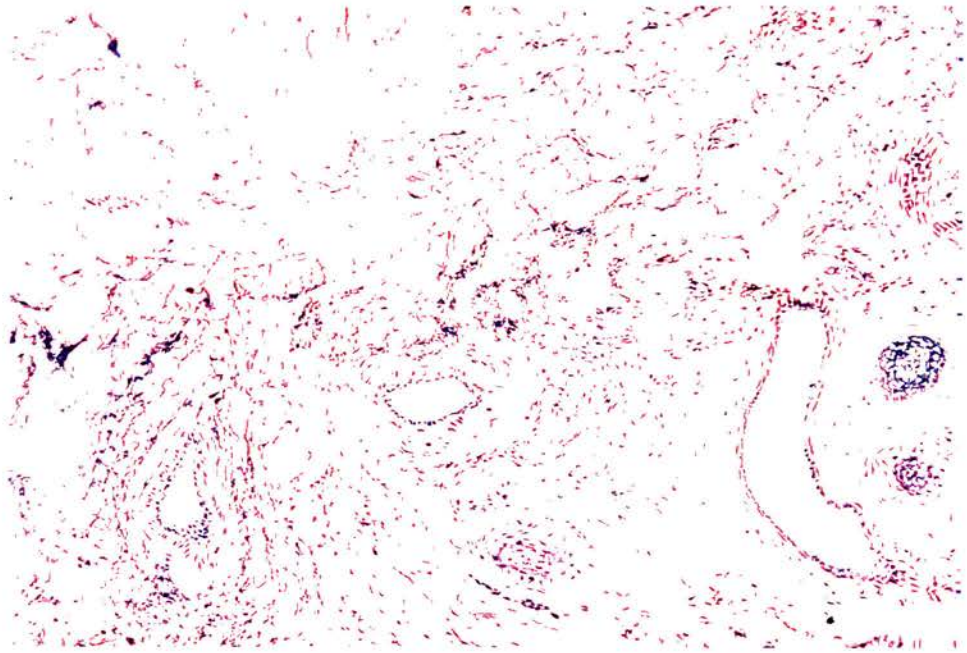


Figure 6.5 A and B

Figure 6.5 Representative histopathological sections of skin from ligand stimulated biopsies

Haematoxylin and eosin stained paraffin sections of normal skin and representative samples of LaM stimulated skin biopsies. Tissues fixed with zinc sulphate fixative. (**A**) showing normal epidermis and (**B**) showing normal dermis with minimal cellular infiltrate. (**C**) showing sPBS stimulated skin at 4 hours with a very mild cellular infiltrate in the dermis. (**D**) showing LaM (200µg/ml) stimulated skin at four hours with a marked inflammatory cellular infiltrate in the dermis.

E



F

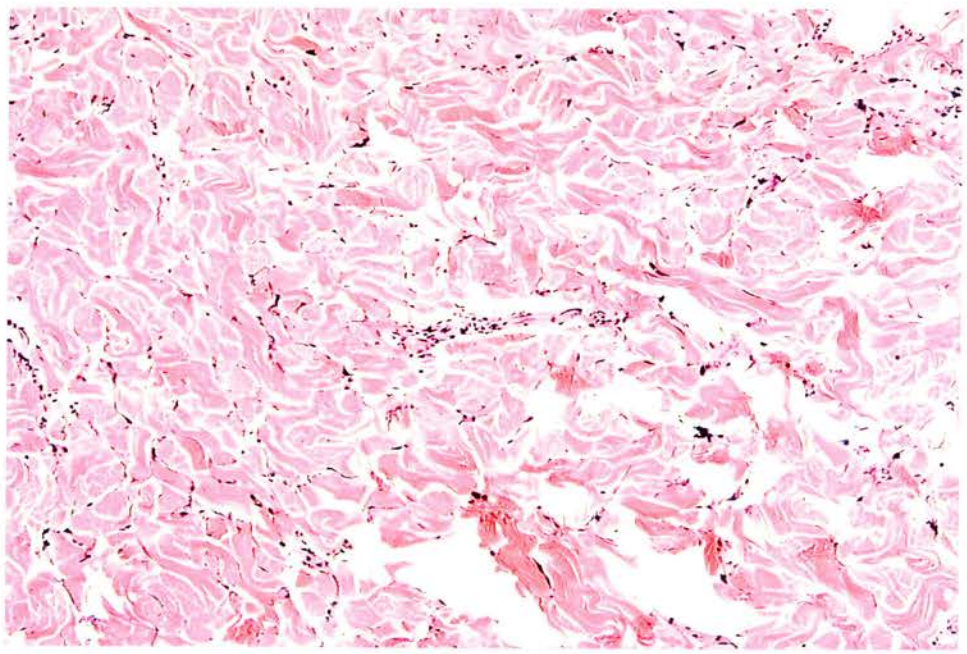
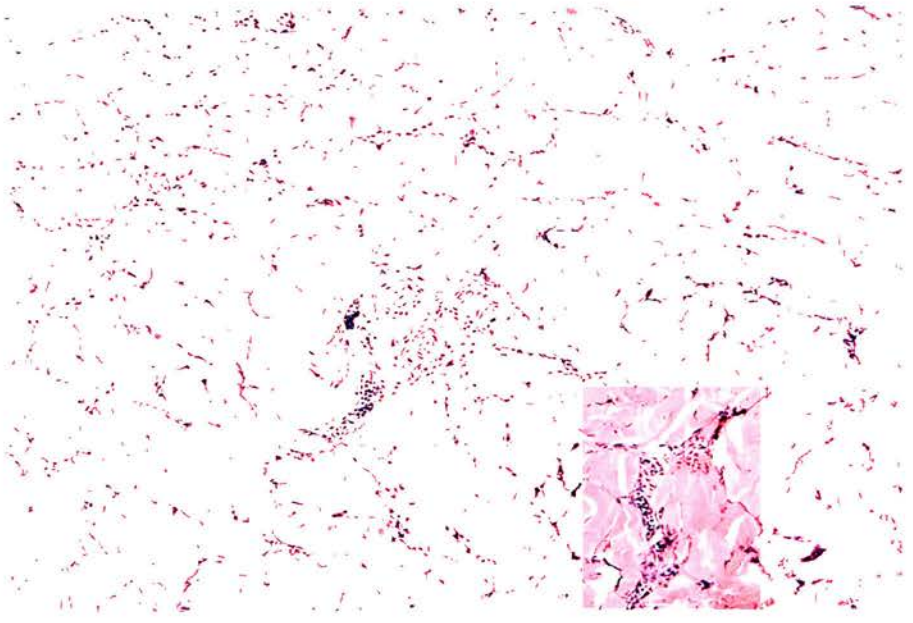


Figure 6.5 E and F

G



H

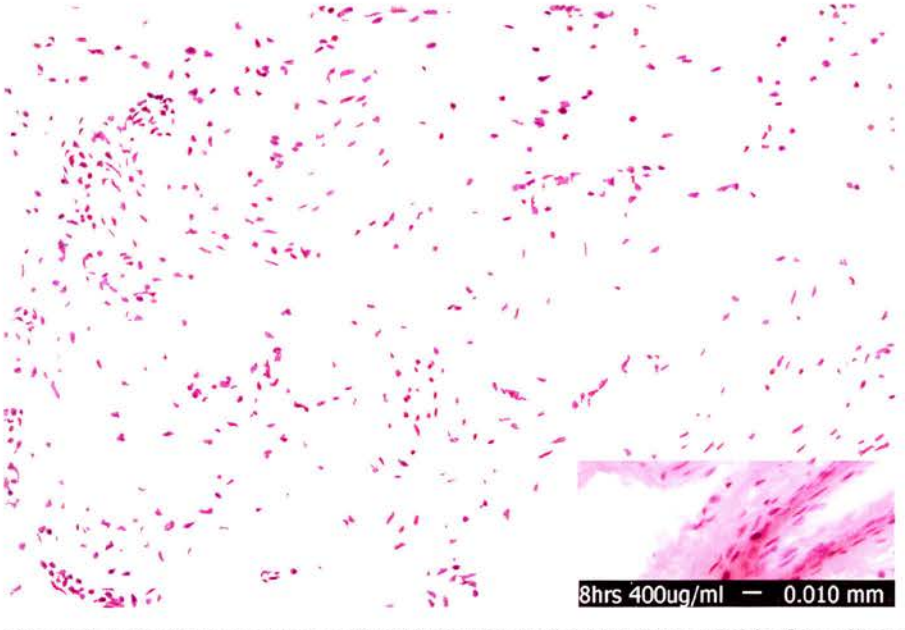


Figure 6.5 Further representative histopathological sections of skin from ligand stimulated biopsies

Haematoxylin and eosin stained paraffin sections of representative samples of LaM stimulated skin biopsies. Tissues fixed with zinc sulphate fixative. (**E**) showing LaM (400µg/ml) stimulated skin at four hours with a marked inflammatory cellular infiltrate in the dermis (100 x magnification). (**F**) showing LaM (200µg/ml) stimulated skin at eight hours with a marked inflammatory cellular infiltrate in the dermis (100 x magnification). (**G**) and (**H**) showing LaM (400µg/ml) stimulated skin at eight hours with a marked inflammatory cellular infiltrate in the dermis (100 x magnification and 200 x magnification respectively).

Intradermal injection with the three different doses of LaM in sPBS generally resulted in similar response curves in the selected PRRs studied over an eight hour period (Figure 6.4). In seven of the 10 genes selected for the dosage trial, the control samples gave a lower expression of the PRRs but there was always a parallel increase over time and a drop in PRR expression after four hours. Most of the PRRs examined seemed to show a peak expression at 4 hours and then a drop in PRR expression by eight hours. For all PRRs examined, other than TLR3, in the LaM-stimulated skin the expression rose to greater than three fold compared to normal unstimulated skin. For TLR3 there was no discernible pattern or trend in the expression between the three different doses and the control and the levels of expression were mostly lower than normal skin. MyD88 expression also rose in the three LaM dosages to levels much higher than the sPBS treated control skin and untreated skin. There was however a slight increase in expression in sPBS treated control skin that was lower but paralleled that of the LaM treated group.

Histopathological examination demonstrated a clear difference in inflammatory cellular infiltrate between the LaM treated skin biopsies and the control sPBS treated skin (Figure 6.5). The LaM treated skin biopsies had very high cellular infiltrates increasing over time while the sPBS control biopsies had only very mild cellular infiltrates at all time points studied.

6.4.2 LaM stimulated skin experiment

Based on the findings from the preliminary dosage test (Section 6.4.1) where the three dosages gave comparably higher expression of most PRRs it was decided to use a lower concentration and volume to mimic a more likely natural low-dose infection situation and to reduce the tissue damage resulting from high infusion volumes. A dosage of 50µl of 50µg/ml LaM in sPBS per injection site was adopted, and four sheep were utilised with five time points (1hr, 2hr, 4hr, 8hr, and 24hr). As a control, 50µl of sPBS were similarly injected at each time point. Skin biopsies were again collected as outlined in the Chapter 2 (Section 2.2.6) and PRR gene expression evaluated for using qPCR. Figure 6.6 shows transcripts expression of a selected panel of PRRs from one animal.

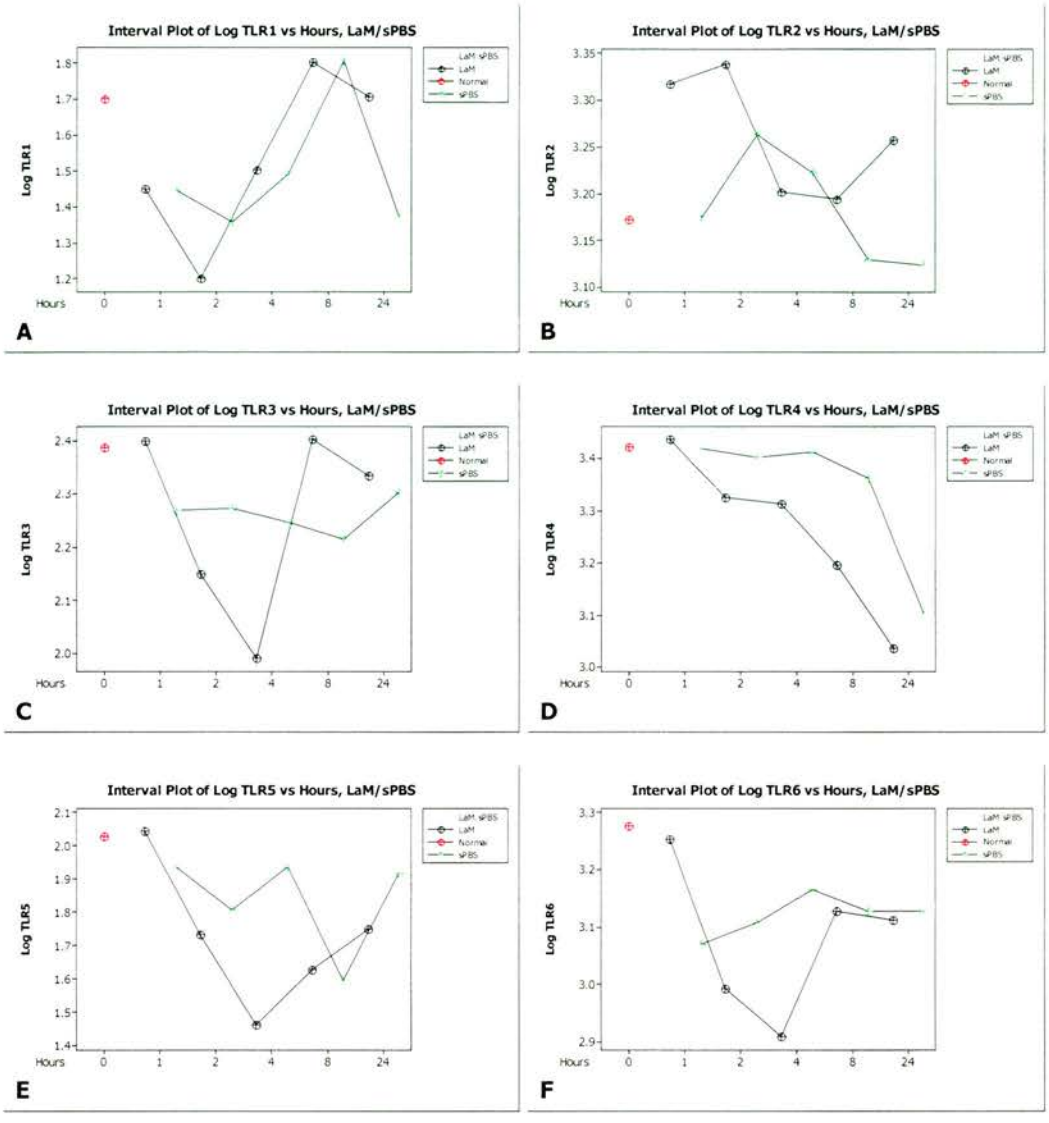


Figure 6.6 Graphical representation of PRR expression over 24 hours in skin stimulated with 50µl (50µg/ml) LaM intradermally

RT-qPCR analysis of TLR1 (**A**), TLR2 (**B**), TLR3 (**C**), TLR4 (**D**), TLR5 (**E**) and TLR6 (**F**) mRNA expression levels in skin biopsy samples treated by intradermal injections of 50µl (50µg/ml) LaM in sPBS. Data are expressed as mean values of duplicate samples normalized Log_{10} (copy number per qPCR reaction) of each PRR over time. 50µl of sPBS was injected over the same time periods as a control. The black circles on the graphs represent the mean PRR expression in LaM treated skin at each time point and the green circles representing the mean PRR expression in control sPBS treated skin. Time point 0 represents the mean mRNA expression from untreated skin.

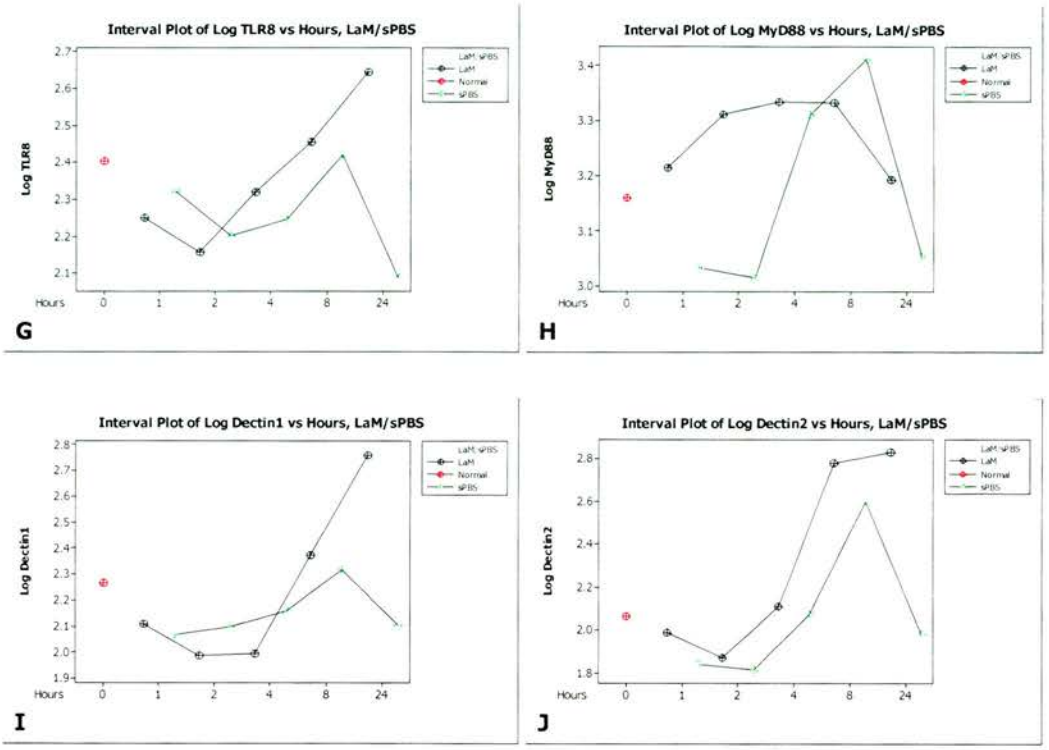


Figure 6.6 Graphical representation of PRR expression over 24 hours in skin stimulated with 50µl (50µg/ml) LaM intradermally

RT-qPCR analysis of TLR8 (**G**), MyD88 (**H**), dectin-1 (**I**) and dectin-2 (**J**) mRNA expression levels in skin biopsy samples treated by intradermal injections of 50µl (50µg/ml) LaM in sPBS. Data are expressed as mean values of duplicate samples normalized Log_{10} (copy number per qPCR reaction) of each PRR over time. 50µl of sPBS was injected over the same time periods as a control. The black circles on the graphs represent the mean PRR expression in LaM treated skin at each time point and the green circles representing the mean PRR expression in control sPBS treated skin. Time point 0 represents the mean mRNA expression from untreated skin.

These results generally showed an unexpected decrease in PRR expression after 50µl (50µg/ml) injection of LaM. PRR expression levels in treated biopsies fell to levels lower than untreated skin with most levels subsequently rising after the two or four hour point. These results did not show marked differences with the sPBS which was meant to be the control and in some cases the sPBS treated skin had higher PRR expression than the LaM treated skin (Figure 6.6 C, D, E and F). There were however differences in cellular infiltrates between the sPBS treated and LaM treated skin samples (data not shown) similar to the ones observed in the dosage trial experiment.

Due to results from the 50µl (50µg/ml) dose experiment being markedly different from the initial dosage test experiment it was decided to discontinue further real time PCR analysis of these samples and concentrate on other possibly more fruitful experiments.

I concluded that the dose that was chosen, in an attempt to mimic a low dose physiological infection model, was below the threshold to significantly activate PRRs in a reproducible manner.

6.5 Analysis of Mutations: TLR2 Exon2 and CARD15 Exon 11

Mutations in ligand binding sites of receptors can lead to defective ligand recognition, while mutations in the intracellular region may lead to defective signalling. Several SNPs (that predispose to mycobacterial diseases and intestinal inflammatory disease) in humans and murine models have been described in the literature. Using direct sequencing, I investigated TLR2 exon2 and CARD15 exon11 for equivalent SNPs in ovine paratuberculosis ileum from the three clinical forms of JD.

6.5.1 Mutation analysis of the partial sequence of TLR2 Exon2

Table 6.3 shows the location and distribution in the JD clinical types of the SNPs that were identified from the partial sequencing of TLR2 from 40 cases of paratuberculosis.

Change		Conserved	Frequency in ovine paratuberculosis type		
Nucleotide	**Amino acid**		**Asymptomatic**	**Multibacillary**	**Paucibacillary**
$A^{182}C$	Asp → Ala	No	2/12	3/16	3/12
$C^{1245}G$	Silent	N/A	5/12	4/16	3/12
$T^{1257}G$	Silent	N/A	3/12	1/16	2/12
$T^{1516}C$	Leu → Phe	No	4/12	5/16	4/12
$T^{1545}C$	Silent	N/A	3/12	2/16	3/12
$T^{1563}C$	Silent	N/A	7/12	3/16	4/12
$C^{1740}T$	Silent	N/A	6/12	3/16	4/12

Table 6.3 Summary of ovine TLR2 SNPs from ovine paratuberculosis ileum tissues

Table showing the distribution of the SNPs found from direct sequencing of the partial sequence of ovine TLR2 exon2. Nucleotide numbering based on submitted sequence of ovine TLR2 (Accession AM117123). Abbreviation: N/A = not applicable.

Sequencing data of the partial sequence of TLR2 exon2 demonstrated two amino acid changing SNPs located at position 182 and 1516 (see Figure 6.7) relative to the submitted sequence of ovine TLR2 (Accession AM117123). Five other SNPs were identified in the sequence, but they were silent mutations. Heterozygosity was also observed in various DNA samples in the SNPs outlined in Table 6.3. Representative chromatogram tracings of the amino acid changing SNPs are in Appendix X.

The distribution of all the SNPs identified in this study was however, almost equally distributed between the three clinical forms and not skewed towards any one group.

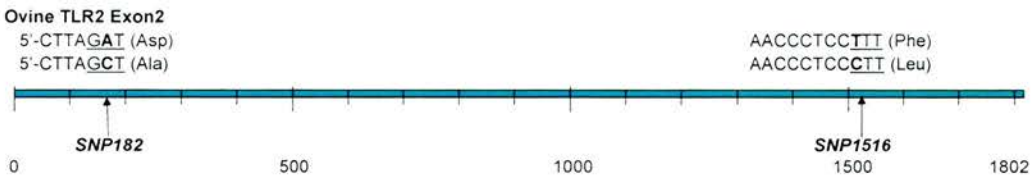


Figure 6.7 Ovine TLR2 amino acid changing SNPs

Position of the locus of the two amino acid changing SNPs identified within the sequenced region of the sheep TLR2 Exon2 (Genbank Accession AM117123) SNP182 Asp/Ala and SNP1516 Leu/Phe. On the sequence fragments shown, the bold represents the nucleotide changes and the underlined nucleotides represent the codon.

For further verification of the two amino acid changing SNPs of the TLR2 gene, tetra-primer amplification refractory mutation system (tetra-primer ARMS) assays were developed as outlined in Section 2.8.4.

6.5.2 Tetra-primer Amplification Refractory Mutation System (tetra-ARMS)

The expected product sizes from the SNP 182 tetra-primer ARMS PCRs were; outer common primers 194 bp, A allele 145bp, C allele 110bp. Homozygous alleles would be identified by two bands (common primers band and the allele specific band), thus the A allele would have a 194bp band and a 145bp band, whilst the C allele would have a 194bp band and a 110bp band. A heterozygous sample would exhibit all three bands (see Table 2.9 in Section 2.8.2).

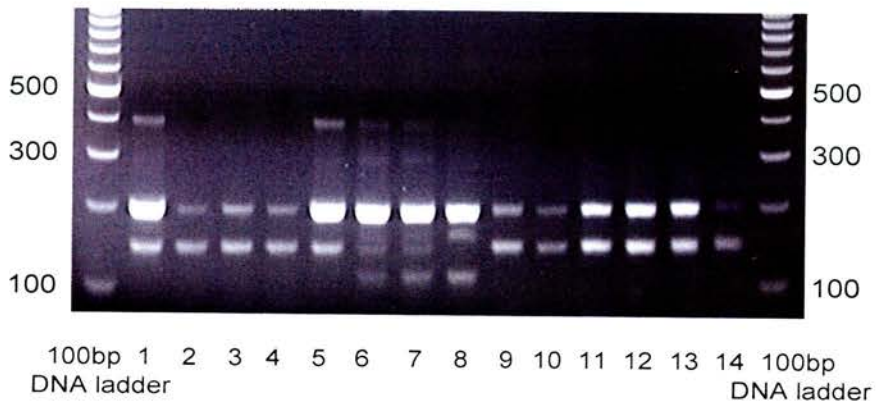


Figure 6.8 2% Agarose gel showing SNP182 tetra-ARMS PCR products

Agarose gel electrophoresis of tetra-primer amplification refractory mutation system PCR amplicons visualized with ethidium bromide staining on 2% TAE agarose gels with 100bp DNA ladder. Lanes 1-5, 9-14 showing the A allele; lane 6 and 7 showing an A/C heterozygous and lane 8 showing a C allele.

Tetra primer ARMS of SNP $A^{182}C$ showed the expected bands for the different alleles but there was evidence of non-specific bands as well. Lane 8 of Figure 6.8 representing the C allele had evidence of a non specific band just below the 194bp outer band but larger than the A allele band. A allele DNA samples demonstrated two distinct bands of the expected size for most samples. Non-specific bands were more evident where there was a stronger signal for the outer common product indicating that it could be due to higher concentration of starting amount of DNA template. Assay optimization would probably eliminate non-specific banding and increase the usefulness of this assay.

6.5.3 Mutation analysis of CARD15 Exon11

CARD15 is a LRR containing PRR and mutations in the LRR region have been described associated with the human inflammatory bowel condition, Crohn's disease (Ogura *et al.*, 2001). Crohn's disease has similar clinical immunopathological presentation as ruminant Johne's disease and, although controversial, some researchers consider *Map* as the causative agent of the disease (Greenstein, 2003; Naser *et al.*, 2004).

No CARD15 exon 11 SNPs were identified in the forty samples of genomic DNA derived from clinical JD cases that were examined. However, an interesting observation was the presence of a 5 base insert in the intron portion of the ovine sequence (Accession AM117124) consistently seen when compared to the closely related bovine genomic DNA sequence (Accession AY518747) (Figure 6.9). The importance of this difference in bovine and ovine CARD15 between these two closely related species is unknown.

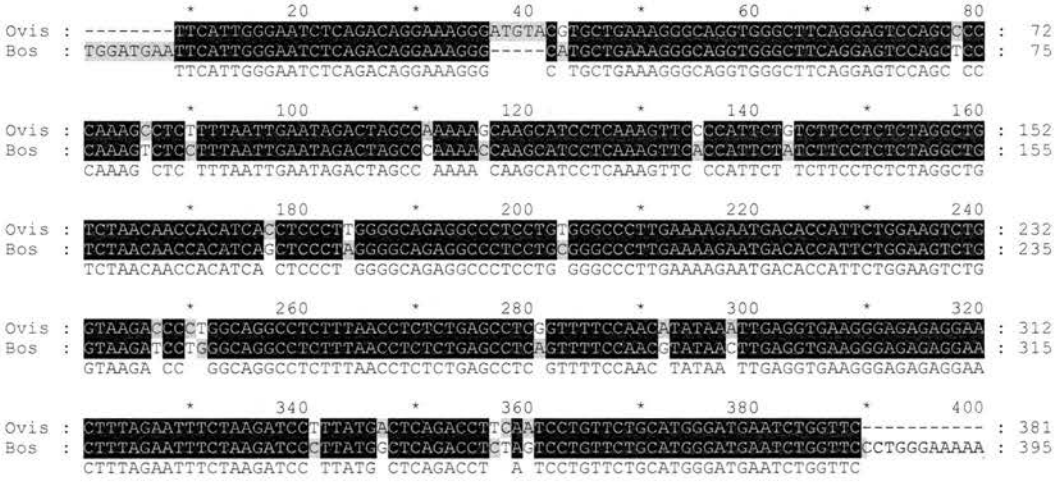


Figure 6.9 Alignment of ovine and bovine CARD15 Exon 11 genomic DNA nucleotide sequences.

ClustalW alignment and Gendoc® presentation of the ovine CARD15 exon11 genomic DNA sequence (Accession AM117124) compared to the bovine CARD15 exon11 genomic DNA sequence (Accession AY518747) showing the location of the 5 base insert in the intron sequence part of the sequences (Ovine CARD15 exon lies between position 149-232).

6.6 Discussion

Johne's disease is primarily an enteric infectious disease and the type of immune responses mounted in the gut play a critical role in the determination of the outcome of natural infection. The gastrointestinal tract also has a large commensal microbiota and immune mechanisms in the gut thus have to comprise a steady balance between immune reactivity and homeostasis. Little work has been done to dissect the exact innate immune mechanisms involved in JD pathogenesis, but extrapolating from work on other mycobacterial diseases such as tuberculosis and leprosy, supports the premise that the innate immunity and an effective cell-mediated immune response is critical for host defence against paratuberculosis . The ability of the innate immune system to recognize pathogens is mediated by PRRs and engagement of these PRRs activates host defence mechanisms that include the regulation of phagocytosis, maturation of DCs and specific antimicrobial action. These signalling pathways are thus able to generate effector responses that may include either Th1 or Th2 responses. In the case of mycobacterial infections, Th1 responses tend to be protective and Th2 responses tend to be ineffective. The role played by PRRs in chronic infections such as JD is poorly understood. I undertook this study to elucidate the role played by selected PRRs in the determination of clinical outcome to *Map* infection in sheep. This study has shown the role of various PRRs in the three different clinical forms of JD.

Differences exist between the three clinical types in lymphocyte distribution (Little *et al.*, 1996) and this has a bearing on the differential PRR expression since different immune cells are known to exhibit different levels of PRRs (Dasari *et al.*, 2005; Taylor *et al.*, 2002; Werling *et al.*, 2006; Zarember and Godowski, 2002). Gamma delta T (γδT) cells play role in improved immunity to ruminant mycobacterial infections (Beard *et al.*, 2000; Kennedy *et al.*, 2002). Beard and colleagues (Beard *et al.*, 2000) showed that γδT cells increase in the ileum during early *Map* infection, prior to the onset of gross pathology. The latency of *Map* infection in calves has been attributed to the high level of γδT cells during this stage in their development (50-

70% of peripheral blood lymphocytes). Kennedy and co-workers (Kennedy *et al.*, 2002) demonstrated a role of γδT cells early in bovine *M. tuberculosis* infection, where animals experimentally depleted of γδT cells showed lower IFNγ production possibly leading to poor Th1 development. Most studies show an increase in the proportion and absolute numbers of macrophages and CD4+ T cells and CD4+CD25+ Treg cells infiltrating the *Map* infected ileum (Bassey and Collins, 1997; Weiss *et al.*, 2006) although in contrast Koets and colleagues (Koets *et al.*, 2002) reported a decrease in CD4+ cells and an increase in γδT cells. Multibacillary JD also has a higher content of macrophages and eosinophils in the garanulomatous lesions, while the paucibacillary form has a high CD4 T cell infiltrate in the ileal lesions.

Granuloma formation is an important protective mechanism in mycobacterial infections that limits the spread and dissemination of bacteria. Contrary to earlier belief that the granuloma walls form a permanent barrier to the animals body and thus immune system, it has been revealed that a granuloma is in a constant state of communication with the immune system and may be modified (Chiu *et al.*, 2004). The granulomata are likely sites where innate and adaptive immune responses merge, regulated by APCs such as dendritic cells. Changes in PRR expression in the granuloma lesion may thus reflect the mild immune sampling being permitted by the granuloma immune exchange mechanisms. As PRRs are critical in the initiation of the early immune mechanisms by directly sensing PAMPs, their expression in advanced mycobacterial granuloma may be lower than non-granulomatous disease.

No differences in TLR1 expression were demonstrated by this study in the clinical forms of JD and the control ileum.

This study showed that TLR2 mRNA expression was significantly higher in multibacillary JD than in asymptomatic sheep. The higher TLR2 signalling in the multibacillary form could have two possible broad explanations; it may exclusively reflect the increased stimulation of TLR2 in response to the high *Map* infiltrate in multibacillary JD ileum. However, if the TLR2 expression is proportionally higher compared to the paucibacillary and asymptomatic JD, one would logically expect it to be proportionally protective and therefore to be no differences in disease outcome

and pathology between the three clinical forms. The higher TLR2 expression in the multibacillary form of JD would be expected to lead to an elevated Th1 type response that would be able to eliminate the *Map* or limit the dissemination of infection. This however, is not the case as evidenced by the severe Th-2 mediated pathology and *Map* infiltration in the multibacillary JD. The other possible explanation is that the TLR2 signalling is responsible for the differences in pathology by some other mechanism other than that of a proportional antigen recognition leading to a proportional protective immune response. In this case, the elevated TLR2 expression would be directly responsible for the increased pathology seen in multibacillary JD, that is known to result from a predominantly Th2 type immune response. The high TLR2 in the multibacillary form of disease may also reflect the high macrophage content in this form of disease and macrophages have high TLR2 expression (Flo *et al.*, 2001; Muzio *et al.*, 2000; Zarember and Godowski, 2002).

The role of TLR2 activation and signalling in driving either pro-inflammatory or anti-inflammatory responses or Th1/Th2 polarization is controversial. Disparate reports have been published that indicate that TLR2 signalling can initiate both Th1 and Th2 cell differentiation (Dillon *et al.*, 2004; Re and Strominger, 2004; Redecke *et al.*, 2004; Sieling *et al.*, 2003; Thoma-Uszynski *et al.*, 2000). It has been shown that different TLRs lead to pathogen-specific pro-inflammatory Th1 type immune responses, but there has been growing evidence that TLR2 signalling may lead to an anti-inflammatory and a predominantly Th2 type response (Dillon *et al.*, 2004; Re and Strominger, 2004). The significantly higher expression of TLR2 in the multibacillary JD would be explained by this although it goes against the logical explanation that TLR2 is needed for the recognition of mycobacteria by the immune system. TLR2 driven Th2 polarized immune response to *Map* in the multibacillary JD would best explain the findings of this study. This could be further exemplified by the fact the TLR2 signalling has been shown to be used by some pathogens to evade and/or inhibit immune responses by promoting ineffective Th2 responses and directly suppressing Th1 responses (Netea *et al.*, 2004; Re and Strominger, 2001; Re and Strominger, 2004). Th2 responses mediated by TLR2 may also arise from Heat shock protein (Hsp) initiated signalling in Tregs (Zanin-Zhorov *et al.*, 2006) and Hsp

has been shown to be released during tissue damage which may be the case with multibacillary JD. More specifically, via TLR2, *Map* was shown to inhibit IFN-γ signalling via STAT 1 beta expression (Alvarez *et al.*, 2003) as has the suppression of IL-12 expression via c-Fos by Pam3Cys, a synthetic TLR2 ligand (Agrawal *et al.*, 2003) and schistosome egg antigen (Dillon *et al.*, 2004). These actions inevitably lead to Th2 type immune responses. The paradoxical role of whereby TLR2 signalling is able to drive both Th1 and Th2 immune responses has been shown by other workers (Mancuso *et al.*, 2004).

Recently, TLR2 signalling has been shown to have an effect on Treg function (Liu *et al.*, 2006; Sutmuller *et al.*, 2006) and this may have a bearing on immune effectiveness of such a pathogen driven response and on the eventual disease outcome. During early infection, TLR2 signalling was shown to initially expand the Treg population whilst abrogating their suppressive effect. After the clearance of the infection, the suppressive effect of the expanded Treg population returns to prevent excessive inflammation and achieve homeostasis. This is postulated to be a protective mechanism, but in relation to *Map* infection, any disturbance in this finely balanced homeostatic mechanism would probably lead to the increased proliferation of *Map* present in the macrophages when the expanded Treg population starts to express suppressive effects again.

Map survival may be further enhanced by the immune evasion in a TLR2 dependent mechanism. TLR2 dependent inhibition of macrophage expression of MHC II has been described (Noss *et al.*, 2001) during antigen processing. This inhibition occurs late during infection and may promote the intracellular survival of mycobacteria in macrophages leading to chronic disease. In the case of the multibacillary JD, this may then inhibit effective CD4 T cell driven immune responses and could promote the proliferation and dissemination of *Map* in this form of disease.

Other than being involved in mycobacterial recognition, TLR2 expression in the mutilbacillary form of ovine JD could be involved in the promotion of antigen capture by cells of the intestinal epithelium. This role has been shown by Chabot and colleagues (Chabot *et al.*, 2006) who showed that in mice there is an increased transepithelial transportation of microspheres by M-cells in a TLR2 dependent

mechanism. A higher antigen capture and transcytosis would consequently be responsible for the higher *Map* infiltrate seen in the lamina propria of multibacillary JD with the higher TLR2 expression.

The findings in this study are however in sharp contrast with the findings by other workers working with DCs and leprosy in humans. Thoma-Uszynski and co-workers (Thoma-Uszynski *et al.*, 2000) found that TLR2 activation on DCs led to increased IL-12 and not IL-10 which would preferentially promote a Th1 type response. In addition, Krutzik and colleagues (Krutzik *et al.*, 2003) found a greater expression of TLR2 in paucibacillary leprosy by immune staining, while my data show greater mRNA expression in multibacillary ovine JD ileum. The same authors also found that the TLR2 signals co-localized with CD14 expression indicating the presence of monocyte/macrophage lineage cells where there was high TLR2 expression. The current study showed a significantly higher CD14 mRNA expression in multibacillary JD. This is most probably due to the infiltration of macrophages into the lamina propria of the infected ileum in response to *Map* infection (see Figure 6.1). It is known that *Map* infected ileum has a higher proportion of CD14+ macrophages than uninfected ileum in cattle (Weiss *et al.*, 2006). The differences could be that the ileum normally has very low CD14 and resident macrophages are known to have low expression of PRRs such as CD14 and TLR4 (Abreu *et al.*, 2001; Smith *et al.*, 2001; Smythies *et al.*, 2005) and this was demonstrated in this study by the significantly lower CD14 expression in the control ileum (Table 6.2M).

The other possible difference with the TLR2 expression in leprosy (Krutzik *et al.*, 2003) and JD could be due to the differences in immune mechanisms of the skin and that of the gastrointestinal tract mucosae. The skin has high regenerative and repair capabilities and thus is be able to tolerate acute inflammation without very severe consequences whilst in the ileum severe inflammation would lead to a breach in the integrity of the mucosal epithelium and would usually have very severe hyperinflammatory consequences. This heightened immune reactivity is partly due to the higher number of microrganisms in the gut and poor regenerative capacity with lesions that would heal very poorly and tend to form erosive lesions. Thus, whereas skin would tend to promote and tolerate a DTH inflammation, the GIT would tend to

promote Th2 type immune responses with dampened inflammation. Krutzik and co-workers (2003) also showed a concurrent higher expression of TLR1 in leprosy possibly suggesting a heterodimerization. Ovine JD TLR1 expression in this study was not significantly different in the different ileum tissues.

On the other end of the spectrum looking at the function and necessity of TLR2 in mycobacterial pathology, some workers have demonstrated that TLR2 signalling may not be necessary for protective immune responses to mycobacterium. Jang and colleagues (Jang *et al.*, 2004) showed that *M. tuberculosis* in murine DCs tends to produces IL-6 and IL-10 via TLR2 and 4 and also that IL-12 can be produced by these cells independently of TLR2. This would tend to strengthen the case that the TLR2 signalling would have immuno-modulatory effects via Th2 polarization, while Th1 responses may occur independently of TLR2 engagement. Working with TLR2, TLR4 and TLR6 knock out mice, Nicolle and associates (Nicolle *et al.*, 2004) concluded that these PRRs are not required for long term control of *Mycobacterium bovis* BCG infection. Drennan and colleagues (Drennan *et al.*, 2004) were able to show that TLR2 deficient mice were able to generate an antigen-specific cell mediated Th1 type immune response to aerosol *M. tuberculosis*, although it was not protective and the mice succumbed to infection. It thus brings into question when the need for the TLR2 begins and ceases, since it is known to be required for the recognition of *Mycobacterium,* and *Mycobacterium* granulomata are understood to be active interfaces with the immune system; constantly providing stimulus to the host's immune system.

Although it has been traditionally believed that TLR signalling preferentially initiated Th1 immune responses, it is becoming increasingly evident that this may not always be the case. TLR signalling is known to drive adaptive immune responses and it was initially proposed to exclusively favour Th1 development (Schnare *et al.*, 2001) and later shown to also drive Th2 responses, but now known to be capable of activating both. Th2 development was thought to occur as a default pathway in the absence of TLR engagement and signalling. Netea and co-authors (Netea *et al.*, 2005) proposed that Th2 type responses that result from the activation of TLR2 are important for the resolution of inflammation and return to homeostasis. Other than

the intensity of expression of TLR2 in advanced disease, it has been shown that the regulation, time of onset and the duration of PRR expression all seem to have a critical effect on the progression of leprosy in humans.

A compromise would be the view that is growing in acceptance, that prolonged low-dose stimulation of TLRs promotes Th2 whilst acute (and/or initial) stimulation would evoke a protective Th1 type response as advocated for by numerous workers (Eisenbarth *et al.*, 2002; Mancuso *et al.*, 2004). Colonic IEC have been shown to have very low expression of TLR2 (Melmed *et al.*, 2003) and then increased expression in disease. This study with the different clinical forms of JD did not show marked difference between the 'normal' control animals and the asymptomatic animals.

The disparate role of TLR2 in *Map* pathogenesis may be reconciled by concluding that acute and early TLR2 signalling leads to Th1 type responses and late, chronic and/or low key TLR2 signalling leads to Th2 type response; this begs the question as to what exact intracellular mechanism tips the balance? It may be possible that when the *Map* infection reaches a particular threshold it would tend to promote Th2 responses by default. It may be a dysfunction in the immune modulation effect and timing in relation to *Map* bacterial load at the time that the immune modulatory mechanisms are taking effect. The other possibility is that the causative agent in the three clinical forms is different and that each clinical form has a different genotype with different virulence, and consequently leading to different pathology. Manca and associates (Manca *et al.*, 2001), demonstrated that different clinical isolates of *M. tuberculosis* have varying virulence, ascribed to strain-specific abilities to drive Th1 immune responses. Janagama and colleagues (Janagama *et al.*, 2006), demonstrated *Map* genotype-dependent differences in virulence in an *in vitro* bovine macrophage model. They showed that certain *Map* strains had higher intracellular survival and in macrophages and a significantly up-regulated IL-10 expression.

No differences in TLR3 expression were observed between the three clinical forms of JD, all of which show significantly higher levels than control sheep. A possible explanation of this finding could be that TLR3 expression is linked to the intracellular release of nucleic acids due to tissue degradation during *Map* infection.

Endogenous, mRNA freed following cell death or degradation has been shown to be a ligand for TLR3, TLR7 and TLR8 (Kariko *et al.*, 2004). This would explain the higher TLR3 and TLR7 expression in asymptomatic JD and multibacillary JD respectively, compared to normal control ileum. Endogenous ligands such as RNA are normally sequestered from the immune system and may become accessible due to apoptosis or cell death.

The significant difference in TLR8 expression between the multibacillary ileum and both paucibacillary and asymptomatic ileum came as a surprise finding since TLR8 is strongly reported to have guanosine and uridine-rich viral ssRNA as the natural ligand. Other synthetic ligands include imidazoquinolone, an antiviral/anti-tumour compound. I speculate several possible mechanisms to explain the higher TLR8 expression in multibacillary JD than the other forms. It could be a regulatory mechanism to counteract the high proinflammatory cytokines that have been shown to be released during *Map* infection in ruminants. Zarember and Godowski (2002) showed that PMA differentiated THP-1 cells increased expression of TLR8 by forty fold in response to IFNγ treatment. The high TLR8 expression in the multibacillary ileum may be in response to the high IFNγ that is present during *Map* pathogenesis. However, paucibacillary sheep are known to express more IFNγ than the multibacillary form (Burrells *et al.*, 1999) but the expression of TLR8 was higher in multibacillary ileum than pauci and the levels between pauci and asymptomatic were comparable. TLR8 synergy with other TLRs has also been shown to promote a Th1 type response in DCs (Napolitani *et al.*, 2005) and the high TLR8 expression in multibacillary JD may be an attempt to revert the immune phenotype into a protective Th1 from the ineffective Th2 type that predominates in multibacillary JD. However, a more plausible explanation may be that the TLR ligand may be the self-nucleotides that result from tissue and cell degradation acting as auto antigens. Numerous workers have shown that self RNA may activate TLR8 (Vollmer *et al.*, 2005).

Further, TLR7 and TLR8 have common ligands such as ssRNA from viruses and imidazoquinolones (Heil *et al.*, 2004) and would therefore be expected to have comparable expression patterns. However, the TLR7 expression was not significantly

different in the three clinical forms of paratuberculosis. Multibacillary JD however had a significantly higher expression of TLR7 than control ileum. This may implicate self nucleic acids as the ligand in the clinical forms that leads to higher expression in response to tissue damage. Recently, functional differences between TLR7 and TLR8 have been demonstrated (Gorden *et al.*, 2005), thus the expression patterns of this two TLRs may differ. Recently, TLR8 signalling has been shown to reverse regulatory CD4+ T cells (Tregs) function (Peng *et al.*, 2005) and this could have significant implications to disease outcome. Regulatory CD4+ T cells are known to modulate the inflammatory response (Wraith *et al.*, 2004) of a host towards commensals and opportunistic infections (Caramalho *et al.*, 2003). Tregs have the ability to suppress a host's immune function and also suppress autoimmunity. Thus, when CD4+ Tregs are suppressed, for example by TLR8 signalling, this may lead to autoimmune disease (Hori *et al.*, 2003). An increase in infiltration of ileal tissues with CD4+ CD25+ T regulatory cells has also been described in subclinical *Map* infection (Weiss *et al.*, 2006). This increased Treg infiltration may be a protective mechanism of dampening down the initial inflammatory response to *Map* infection as Tregs have been shown by Taams and co-workers (Taams *et al.*, 2005) to suppress the proinflammatory properties of monocytes/macrophages.

Thus I propose that during the pathology of multibacillary JD, there is an increase in macrophages that lead to excessive inflammation to eliminate or contain *Map* infection leading to sustained inflammation over prolonged time periods. Tregs also increase at the inflammatory site to modulate the immune response in an attempt to return to physiological homeostasis. Prolonged Treg activity could lead to an immunosuppressive state, thus the increased TLR8 expression may be due to a negative feedback mechanism towards the Tregs to counteract the immune regulatory effect of Tregs that was initially triggered by tissue destruction, cell death and an increased number of macrophages infiltrating the ileum (as evidenced by the ileal histopathology (Figure 6.1C and D) and higher CD14 expression in the multibacillary from (Figure 6.2M and Figure 6.3M)) and prolonged inflammation. This however does not elucidate the specific ligand during ileal *Map* pathogenesis that leads to the increased TLR8 expression in multibacillary JD. Tissue nucleic

acids following tissue destruction are a strong candidate leading to the increased TLR8 signalling leading to Treg suppression that may explain the hyper-inflammatory pathology in multibacillary JD despite an increase in Treg infiltration that has been described. Immune suppression of the intestinal mucosa during *Map* infection that could be ascribed to Tregs has been reported recently in cattle with subclinical infection (Weiss *et al.*, 2006).

The three clinical forms of JD had statistically comparable expression of TLR9 and TLR10. Clinical forms of JD had comparable levels of TLR10 to control ileum but they had a significantly higher TLR9 expression. Bacterial DNA is the natural ligand for TLR9, and the higher expression of TLR9 in clinical JD may reflect the fact that control ileum has intact ileal epithelium and thus no active subepithelial immune response is being mounted against any bacteria whilst the clinical forms of JD have breaches in the integrity of the epitheliae and are actively responding to various bacterial challenges.

MyD88 is a critical adaptor molecule for the downstream signalling of all TLRs except TLR3. Its engagement leads to intracellular signalling cascades that culminate in immune responses. The minor differences in MyD88 levels of expression observed between the three different clinical forms of JD were rather unexpected. With expected higher inflammatory status and the higher expression of TLR2, TLR4 and TLR8 in the multibacillary JD, a significantly higher expression of MyD88 would have been expected. Though the expression was higher than the asymptomatic JD, the difference was not statistically different. There was a significant difference in MyD88 expression between the asymptomatic and paucibacillary JD which may reflect a more active inflammatory state during this clinical form. The multibacillary JD had slightly lower expression of other TLRs (TLR1, TLR5, TLR6, TLR7, TLR9, and TLR10) than both asymptomatic and paucibacillary and this may explain the lack of large differences in the MyD88 expression between the clinical types. This would be assuming that the overall tissues MyD88 expression is a cumulative total of each TLRs proportional contribution. This study's finding would also concurs with findings by Shi and co-workers (Shi *et al.*, 2003) who found that in *M. tuberculosis*

infection, a host marshals its immune responses in a largely MyD88-independent manner.

CARD15 has been reported to be either a specific intracellular PRR for muramyl dipeptide (MDP), a break down product of PGN, (Girardin *et al.*, 2003) or a negative regulator of TLR2 signalling (Watanabe *et al.*, 2004). This study demonstrated a significantly higher expression of CARD15 in the pauci- and multibacillary JD compared to the asymptomatic JD and control ileum with comparable expression between the asymptomatic JD and the control ileum. The higher CARD15 expression in the paucibacillary and multibacillary forms could be a reflection of the innate immune recognition of the intracellular presence of *Map* in the macrophage infiltrate of these two forms of JD. However, multibacillary JD has a higher macrophage infiltrate than the paucibacillary JD and it would then be expected that it would have a significantly higher CARD15 expression. In spite of this difference in macrophage infiltrate, the expression levels of CARD15 were not statistically significantly different between these two clinical forms of JD. Berrebi and associates (Berrebi *et al.*, 2003) showed that other than lamina propria macrophages, during inflammation, colonic epithelial cells produce copious amounts of CARD15. They also showed that uninflammed colonic epithelial cells express very little or no CARD15. Thus the similar expression of CARD15 between the pauci- and multibacillary sheep could be more likely attributed to epithelial cell expression in response to ileal inflammation and this would also explain the lower CARD15 in the asymptomatic JD.

Ferwerda and co-workers (Ferwerda *et al.*, 2005) demonstrated the synergistic role of TLR2 and CARD15 in the immunity to *M. tuberculosis*. They concluded that TLR2 and CARD15 are independent and non-redundant PRRs for *M. tuberculosis* and that both are necessary for the effective pro-inflammatory responses. However, in this study, despite there being a comparable CARD15 expression, there was a significantly higher TLR2 expression in multibacillary JD than paucibacillary JD. In contrast to the findings of Ferwerda and co-workers, Tada and colleagues found that CARD15 was synergistic with TLR3, 4 & 9 and not with TLR2 (Tada *et al.*, 2005) in producing pro-inflammatory immune responses in cultured DCs.

CARD15 expression has been shown to increase in response to the cytokines TNF-α and IFN-γ (Gutierrez *et al.*, 2002; Iwanaga *et al.*, 2003; Rosenstiel *et al.*, 2003), and the high expression of CARD15 in the pauci- and multibacillary sheep could be in response to the release these cytokines during *Map* infection. This could be in a regulatory mechanism to prevent excessive hyperactivity effects of these potent proinflammatory cytokines on tissues. CARD15 expression has also been shown to increase production of defensins by Paneth cells (Voss *et al.*, 2006), and this may be an attempt to hasten mucosal repair by reducing the access of lumen microbes to the underlying lamina propria. If indeed the high CARD15 expression is due to the cytokines TNFα and IFNγ, and is regulatory, it could be a similar mechanism to the one proposed for the high TLR8 expression in the multibacillary ileum that may also be in response to these cytokines. However excessive CARD15 may also exacerbate the inflammation, in a positive feedback loop, by further increasing proinflammatory gene expression via NFκB.

Taking into account these findings of CARD15 over-expression in the multibacillary form and the postulated role of TLR2 signalling in Th type polarization, I propose the following explanation. Early CARD15 expression would tend to augment Th1 polarization in the absence of concurrent TLR2 expression. The concurrent over-expression of TLR2 would inhibit Th1 polarization due to IL-10 production and promote a Th2 type response as a default. This would explain the differences shown in this study in both TLR2 and CARD15 expression in the paucibacillary and multibacillary forms of JD. This would agree with the conclusion reached by Tada and co-workers on CARD15 synergism with various TLRs (Tada *et al.*, 2005). Then again, there may also be a temporal relationship in this synergism as TLR2 signalling at late time points itself has been shown to lead to a shift towards Th2 immune responses (Zanin-Zhorov *et al.*, 2006) with, for example, endogenously derived ligands. Late CARD15 expression, which may be due to prolonged proinflammatory cytokine release, would similarly lead to Th2 type responses to dampen protracted inflammatory immune responses.

In human blood, the highest expression of CARD15 is seen in $CD14^+$ monocytes $CD15^+$ granulocytes and $CD40^+/CD86^+$ DC populations (Gutierrez *et al.*, 2002). It

would also imply that these CD14+ CARD15+ expressing cells are most likely newly recruited haematogenous monocytes rather than resident macrophages, since resident intestinal macrophages are known to have very low CD14 expression. Extrapolating from this could explain the higher level of CARD15 and the concurrent high level of CD14 seen in multibacillary JD where there is a high infiltration of macrophages compared with the paucibacillary and asymptomatic forms.

The findings of Berrebi and co-workers (Berrebi *et al.*, 2003) of higher CARD15 expression in Crohn's disease mononuclear cells of the colon than in uninflamed colon and the similar finding in this study would further increase the similarities of the immunopathology of Crohn's and JD. It however, does not give any further insight into the exact molecular mechanisms involved in the determination of disease outcome.

This study showed a significantly higher CD14 expression in multibacillary JD compared to asymptomatic JD. Asymptomatic ileum also had a significantly higher expression of CD14 compared to control ileum. These differences are most likely due to CD14 expression by newly recruited extra-mural myeloid derived cells of the monocyte/macrophage lineage since normal uninflamed intestinal mucosal macrophages have been shown to have very low or no CD14 expression (Smith *et al.*, 2001; Smythies *et al.*, 2005). The control ileum in this study showed very low CD14 expression as compared to the asymptomatic JD ileum, and this may show that the presence of *Map* in the asymptomatic cases may have already triggered the recruitment of macrophages into the ileum in response to the pathogen. Asymptomatic JD ileum has a low *Map* burden (as evidenced by the positive IS900 PCRs) but is ZN negative and has no visible pathology. The presence of *Map* would activate macrophages and induce haematogenous monocyte recruitment into the ileum. Control ileum was IS900 negative, implying the absence of *Map*, and this could explain why asymptomatic JD has a markedly higher expression of CD14 than the controls. However, other than macrophages/monocytes, CD14 is also highly expressed on granulocytes (Ziegler-Heitbrock and Ulevitch, 1993). Thus, the increased CD14 levels could be due to monocytes that have recently migrated from

blood into infected tissues due to their chemotactic recruitment after resident APC/epithelial activation and not due to an exclusive increase in the expression of CD14 by resident cells. In a study of inflammatory response mechanisms in Crohn's disease, Berrebi and co-workers (Berrebi *et al.*, 2003) also demonstrated that most of the monocyte/macrophages were located in the perivascular area and this would tend to imply that these cells are being immediately actively recruited.

In terms of pathology in multibacillary JD, the higher level of CD14 could lead to a hyper-responsiveness to LPS in the GIT mucosae (Cario *et al.*, 2000), which would lead to the characteristic type of lesions seen in this type of JD. Underhill and co-workers (Underhill *et al.*, 1999) showed that the cooperation between TLR2 and CD14 led to a marginal increased in *Mycobacterium* induced NFκB activation.

CD14 is reported to recognize LPS in conjunction with MD-2 and TLR4. However, the TLR4 expression levels between the three clinical forms were not significantly different although it was slightly higher in the multibacillary type JD. It would have been of value to look at MD-2 mRNA expression as well in order to determine whether the CD14 expression is purely due to the high macrophage infiltration.

Dectin-1 and dectin-2 are C-type lectins recently identified as PRRs that have been increasingly studied in the area of innate immune recognition of yeasts and antigen uptake. Their precise physiological functions remain to be fully elucidated. Multibacillary and paucibacillary JD had significantly higher expression of both dectin-1 and dectin-2 than did asymptomatic and control ileum. Dectin-1 and dectin-2 expression was comparable between asymptomatic JD and control ileum.

The high dectin-2 expression in the multibacillary form may simply reflect the arrival of activated cells of the macrophage/monocyte lineage as proposed by Taylor and colleagues (Taylor *et al.*, 2005). In an adoptive transfer experiment in mice, Taylor and colleagues (2005) showed that dectin-2 expression at inflammatory sites is due to novel recruited macrophage dectin-2 expression rather than resident macrophages. It would concur with previous literature on other PRRs expressions and cells of the GIT. These publications show that normal intestinal mucosal tissue has very few activated macrophages, with low expression of PRRs, and that in

diseased and inflammatory states of the intestine the macrophages are mostly novel haematogenously derived (Berrebi *et al.*, 2003; Smith *et al.*, 2001; Smythies *et al.*, 2005). This may be corroborated by the findings of Heinsbroek and colleagues (Heinsbroek *et al.*, 2006), who concluded that the alpha isoform of murine dectin-1 is increased in monocytes during the maturation process and reduces over time as inflammation subsides. Gavino and co-workers (Gavino *et al.*, 2005) showed that Con A activated CD4+ T cells had an induced expression of dectin-2. Part of the dectin-2 seen in the clinical forms of JD may be due to the high CD4+ cellular infiltrate that has been documented. Dectin-1 was initially shown to be exclusively expressed by DCs later shown to be only lowly expressed in macrophages. However, since the infiltrates of the multibacillary JD ileum were predominantly macrophages and not DCs, the high level of dectin-1 mRNA would further emphasize the fact that dectin-1 is also expressed in cells other than DCs.

Dillion and co-workers (Dillon *et al.*, 2006), also found that zymosan induces tolerance via Tregs by increasing TLR2 and dectin-1 expression in macrophages. It may therefore be possible that in ovine multibacillary JD, a degree of tolerance to the *Map* was due to the concurrent increased TLR2 and dectin-1 expression observed in this study. Yadav and Schorey (Yadav and Schorey, 2006) showed a specific cooperation of dectin-1 and TLR2 in the activation of macrophages by *Mycobacterium spp* leading to induction of TNFα production. The specific ligand for dectin-1 on mycobacteria is unknown since mycobacteria do not have β-glucans. However, dectin-1 has also been shown to bind on an undefined molecule on T cells (Ariizumi *et al.*, 2000) and thus it does not preclude the possibility that dectin-1 may bind to other molecules other than β-glucans. Similarly, dectin-2 has been proposed to have a yet unkown ligand on CD4+ CD25+ Tregs.

A remote possibility is that the increased dectin expression observed in the clinical forms of JD could be as a result of the exposure of the immune system to commensal and pathogenic yeasts in the gut lumen as a result of the breach in epithelial integrity. These yeasts would lead to immune activation via increased TLR2 and dectin-1 expression. Gantner and colleagues (Gantner *et al.*, 2003) have shown that dectin-1 and TLR2 cooperate to respond optimally to zymosan by producing TNFα via NFκB.

However, heat killed yeasts have been shown to activate DCs towards and Th2 phenotype producing IL-10 (Edwards *et al.*, 2002).

From the findings of PRR expression described in this chapter, I propose the following events for the three different clinical forms of JD with the caveat that PRR expression in chronic and late-stage GIT disease may not represent a classical clear-cut 'text book' role of single individual PRRs, but a summation of their activities. The complexity of the PRR profile in the ileum may also reflect the complex microbiota that can potentially activate GIT innate immune mechanisms in a homeostatic or pathogenic manner.

These data would infer that *Map* pathology is a result of both a change from the protective Th1 to Th2 but is also due to an increase in the local immune reaction possibly due to the ileum epithelial barrier being breached by the pathology and subsequently bringing the GIT microflora in contact with the immune system. This would then result in a hyperinflammatory state that is typified by the lesion in clinical JD cases. It may however bring out the 'chicken and egg' question as to the order and importance of these two proposed mechanisms in relation to the role of PRRs. Thus the PRR transcript expression seen may be responsible for the protective innate sensing of the *Map* and also to the furthering of intestinal pathology.

The dectin-1 and dectin-2 expression possibly represent *de novo* haematogenous recruitment of monocytes into the lamina propria and their maturation into macrophages during the process of heightened inflammation. This is partially corroborated by the increased CD14 expression in the multibacillary form.

TLR2 expression is initially increased in response to recognizing *Mycobacterium* and this drives a predominantly Th1 response. However, over time this continued TLR2 ligation, due to continued *Map* presence and proliferation, changes and starts driving a Th2 response resulting in multibacillary JD. Thus, multibacillary JD may not be as a result of a deficiency in *Map* recognition by TLR2 (as evidenced by the high TLR2 corresponding to the high *Map* in the macrophage infiltrate of multibacillary JD) but due to a yet-unknown switch in the downstream signalling following the TLR2

ligation that leads to the now well-documented switch from a protective Th1 type response to an ineffective Th2 type response.

A concluding remark is that, PRR expression in disease states is very complicated as the immune responses (or needs) are a sum of all the PRRs that are marshalled against a specific pathogen. Because of redundancy in innate immune recognition via PRRs, *Map* would most probably engage multiple PRRs that would result in the differential activation of multiple anti-microbial effector pathways and mechanisms. This extremely complicated nature has been further exemplified by the fact that combinations of different TLR ligands for example lead to unique cytokine milieu and Th type responses and the intracellular signalling pathways would also involve many positive and negative regulatory molecules with very subtle changes in expression patterns leading to specific immune responses. Further work looking at selected critical signalling pathway molecules and negative regulators of TLR signalling (such as SIGIRR, A20) in relation to the different forms of ovine paratuberculosis would be of value to further understand the hyper-inflammatory state (or lack) of the ileum in JD pathogenesis.

Expression of the different isoforms of dectin-1 and dectin-2 is immune-cell specific and tissue specific (Heinsbroek *et al.*, 2006). This study has shown a differential expression of the long form of dectin-1 and dectin-2, thus it would be of value for future researchers to look at whether the short/β isoform of dectin-1 and dectin-2 have different mRNA expression patterns in the different forms of ovine paratuberculosis. This would also be important in order to rule out compensatory expression of the alternative isoform in one clinical form of JD to compensate for differences seen in another clinical from.

It would be of benefit, in future to further breakdown the PRR expression in ovine paratuberculosis to the cellular level in the ileum using techniques such as laser capture dissection. This is because the RNA we have is the overall RNA of intestinal epithelial cells and that of the cells of the lamina propria and strikingly different immune activities may be taking place at the epithelial cell level and lamina propria. Numerous studies have shown that there is a differential expression of PRRs in these

different constituents of the ileum and the differing expression patterns may have a significant bearing to outcome of infection and intestinal immunity.

Mutations in paratuberculosis genomic DNA samples

Screening for mutations in CARD15 exon 11 was carried out because mutations have been described in human Crohn's disease, which has similar immunopathological presentation to paratuberculosis. The first mutation described was the 3020InsC, that results in a truncated protein that is unable to recognise ligands (Ogura *et al.*, 2001). Two more mutations have recently been described ($Arg^{702}Trp$, $Gly^{908}Arg$) that together with the 3020InsC (Leu^{1007}fsinsC) account for 80% of all Caucasian cases of Crohn's disease (Siminovitch, 2006). Where CARD15 mutations exist together with the TLR4 mutation ($Asp^{299}Gly$) this increases genotype relative risk by 81 to 88% than having either mutation separately (Franchimont *et al.*, 2004). Other CARD15 mutations located in the NBD region have been described associated with Blau syndrome but not with Crohn's disease, and it has been postulated that genetic variants in the LRR region are ligand dependent, and that resultant disorders would be in mucosa in contact with bacteria (Miceli-Richard *et al.*, 2001). Spliced variants of CARD15 have also recently been described (Leung *et al.*, 2007) although their role in immunopathology remains elusive. Ethnic differences exist with all described CARD15 mutations; being predominant in Caucasian Crohn's disease patients but absent from Japanese (Sugimura *et al.*, 2003) and Chinese (Leong *et al.*, 2003) Crohn's disease patients.

CARD15 has been mapped to the 16q12 chromosome in humans and this has been identified as the susceptibility locus IBD1 for Crohn's disease. Similarity in pathology between ovine JD and human Crohn's disease would tend to make this a leading candidate in mutation analysis for SNPs that may possibly explain the discrimination of the three clinical forms of JD. The fact that no SNP was identified in exon 11 of ovine CARD15 gene (Accession AM117124)) may be due to the small sample size examined or possibly that the important relevant SNPs may be located elsewhere on the LRR region of CARD15 gene.

TLR2 (as a homodimer or heterodimer) recognizes the largest variety of described PAMPs amongst the TLRs. TLR2 maps to chromosome 17 in the bovine genomic contiguous sequence. Seven SNPs were detected in the ovine TLR2 exon2 from genomic DNA samples derived from the three clinical forms of ovine JD, and of these seven, five were silent and two were amino acid changing. When mapped to our submitted TLR2 exon2 partial sequence (Accession AM117123) these are; $A^{182}C$ and $T^{1516}C$ and bring about an amino acid change from an aspartic acid to alanine and a leucine to phenylamine respectively (Figure 6.7). However, the distribution of these SNPs between the different clinical forms of JD was not significantly different, suggesting that they do not contribute to JD pathological form differentiation. None of these SNPs correspond to any of the susceptibility SNPs described for human TLR2 and mycobacterial diseases (Bochud *et al.*, 2003; Kang and Chae, 2001; Ogus *et al.*, 2004). Recently, Bhide and co-workers (Bhide *et al.*, 2006) described the identification of the TLR2 $Arg^{667}Trp$ mutation associated in *Map* infection in cattle. They were also able to identify the TLR2 $Arg^{753}Gln$ mutation although its contribution to susceptibility was not significant. Like this study, they also found numerous silent mutations within the bovine TLR2 gene that was sequenced. Unlike this study, they had access to genomic DNA from a large study population of 1636 cattle.

Malhotra and co-workers (Malhotra *et al.*, 2005) re-examined the TLR2 $Arg^{677}Trp$ polymorphisms associated with leprosy and concluded that the possible SNPs identified in TLR2 exon2 by Kang and Chae (2001) were possibly due to sequence variations arising from the presence of a highly homologous duplicated sequence (pseudo-gene) from exon3 of the TLR2 gene. This could explain the numerous SNPs seen from the direct sequencing and expose the possible shortcoming using this as a sole method of detecting SNPs.

An attempt was made to develop tetra primer amplification refractory mutation system (tetra-ARMS) assay for SNP validation and to discriminate the two amino acid changing SNPs. Encouraging results were obtained (see Figure 6.8) but this was abandoned due to limitations of time and to difficulties in assay optimization. It would still be a worthwhile avenue to take in conclusively coming up with definite

TLR2 SNPs in any future SNP analysis project. The 3' end of the TLR2 gene was sequenced from mRNA in order to identify the end of TLR2 exon2 and compare the sequences with the putative exon three and see if it could account for the spurious SNPs. The full ovine TLR2 mRNA sequence has recently become available (Accession DQ890157). Aligning the 3' end of this sequence with all the SNPs described here did not give any match that would uphold the impression that the SNPs could be due to a similar sequence on the three prime end (the putative exon3). The TLR2 mutations associated with leprosy described by Kang and Chae (2001) also seem to be ethnically related as they were initially described in Koreans but have not been found in any other ethnic groups.

As this is a new area of science, disparate number of exons for TLR2 in mammals have been reported; two exons (Takeda *et al.*, 2003) or three (Haehnel *et al.*, 2002; Malhotra *et al.*, 2005; Wang *et al.*, 2001) with Haehnel and co-workers proposing that the TLR2 exon 2 is non coding. Moore and co-workers (Moore *et al.*, 2004) did not find any correlation between TLR2 Arg677 polymorphisms with susceptibility to *Staphylococcus aureus* infections.

Due to the limited sample size and the difficulties in ascertaining that the 'asymptomaytic' case would have never developed into clinical cases, it was difficult to exclude the possibility that the results of the mutations seen are frequent anonymous polymorphisms and were observed by chance alone.

In summary, two productive SNPs in TLR2 exon 2 were identified via direct sequencing and partially verified by tetraprimer ARMS. These reported mutations of TLR2 will need validation in large unbiased populations in order to ascribe an accurate risk they pose to predisposition to the clinical forms of ovine JD.

7. Summary Discussion

7.1 Summary

This thesis deals with the expression and immunopathological importance of PRRs in ovine tissues and cells. PRRs play an important role in the maintenance of homeostasis and immune responses against pathogens. Tissue and cellular expression of PRRs has an effect on the type of immune responses elicited and thus has a bearing on the outcome of host-pathogen interactions. A fundamental pre-requisite to fully understand the role played by PRRs in health and disease is the elucidation of their expression in different normal tissues and cell types.

Chapter Three is concerned with the identification of the sheep homologues of the common PRRs and their adaptor molecules. This formed the basis for developing ovine specific quantitative real time PCR assays for the accurate measurement of sheep PRR transcripts used throughout the rest of the study. Chapter Four deals with the characterization of expression patterns of these PRRs in selected adult tissues and a comparative study of their expression in second trimester foetuses and adult spleen and skin. Chapter Five looks at the PRR expression in immune cell subtypes; APCs, DCs and T cells. Consistent with the role that they play in immunity, PRRs were expressed at higher levels in mucosae that are directly exposed to the externum (and thus have a higher risk of pathogen entry) and also in immunologically important tissues such as the spleen and lymph nodes. The skin however, had low PRR expression and I speculate that it may be a homeostatic mechanism to prevent hyperinflammation or may reflect the lack of PAMP stimulation due to the effective barrier by keratinized squamous epithelia. The leukocyte subsets showed diverse PRR expression profiles, with the CD172a^{+} DCs, monocytes and B cells showing the greatest range of PRRs detected.

Diverging from the baseline data obtained from normal tissues and cells, the functional role of PRRs was studied in Chapter Six that examines the role of PRRs in the pathology of ovine paratuberculosis. Mutations have been described associated with mycobacterial diseases and Crohn's disease that might have functional implications on the pathology of ovine paratuberculosis. An attempt to examine more

closely functional aspects of *in vivo* PRR engagement by a specific PAMP was made in the same chapter by carrying out Lipoarabinomannan stimulation of skin and measuring the cutaneous PRR response. Chapter Six of this study clearly demonstrates functional differences in PRR expression between the clinical forms of ovine paratuberculosis that may have implications on the pathogenesis of clinical disease. No disease discriminating mutations were conclusively identified, and disappointing results were obtained with the cutaneous ligand stimulation study.

7.2 Future Work

This study examined PRR expression in immune cell subsets, but due to low starting RNA and resultant low transcripts, quantitative analyses could not be performed between the subsets. Performing this experiment with adequate number of cells and higher concentration and quantity of RNA, or using nested PCR to enable quantitative comparisons to be performed would be a future research area that would yield useful functional data on the innate immune functions and potential of these cells. The *in vivo* stimulation of APCs, such as macrophages and DCs by a specific ligand and examining the resultant PRR expression would be a beneficial follow up experiment to elucidate functional quantitative PRR expression in these cells.

In order to assess the functional role of PRRs to mycobacterial ligands in experimentally stimulated skin, performing this experiment with a proven concentration of LaM or *M. bovis* BCG with the concurrent examination of the PRR expression in the draining superficial lymph node could possibly yield more practical data. This will enable a comparative analysis of changes in stimulated skin and also the lymph node that drains it to which stimulated APCs migrate with antigen.

As other workers have shown the presence of TLR2 SNPs in cattle infected with *Map* from large scale studies, carrying out similar large scale studies with more study subjects examining the full TLR2 gene may show disease discriminating SNPs in ovine paratuberculosis. The development and optimization of SNP specific real time PCR tetra primer ARMS assays would facilitate rapid turnover of such large

numbers of samples since this would be a high through-put method since it obviates the need for gel analyses of amplicons.

7.3 Concluding Remark

In conclusion, I have obtained nucleotide sequences of TLR1-TLR10, MyD88, CARD15, CD14, Dectin-1 and Dectin-2. I have demonstrated that mRNA for these PRRs and MyD88 is expressed in the kidney, lung, mesenteric lymph node, prescapular lymph node, skin, spleen and urinary bladder of normal sheep. I have further demonstrated PRRs and MyD88 in cells of the immune system and a functional role or PRRs in disease as established by the differences in PRR expression in the different form of ovine Johne's disease.

As this 'decade old' renewed area of immunology expands and the importance of PRRs takes more centre stage it would be important to have more thorough mechanistic dissection of the role that PRRs play in disease onset, progression and resolution. As complete species' genomes become available it would also be of value to mine these genomes for all putative PRRs, their accessory molecules, signalling pathway molecules and negative regulators in order to design custom microarrays that would enable simultaneous *in vivo* analysis of the role played by PRRs in these disease situations. This would allow the in-depth understanding of the role that each PRR plays, how specificity is achieved via adaptor molecules, negative regulation, pathway choice and possible interconnection between various aspects of pathways of innate immune recognition.

8. Appendices

8.1 Appendix I

List of Suppliers of Common Laboratory Reagents

Sigma
Fancy Road
Poole
BH12 4QH
UK

Qiagen
Qiagen House
Fleming War
Crawley
West Sussex
RH10 9NQ

Sigma Genosys
Sigma-Aldrich C. Ltd
Homefield Road
Haverhill
CB9 8QP

Roche Diagnostics UK Ltd
Unit2 – The Drove
Newhaven
East Sussex BN9 OAG

New England Biolabs (NEB) UK Ltd
73 Knowl Piece,
Wilbury Way
Hitchin,
Herts
SG4 0TY

8.2 Appendix II

Enrichment of Dendritic Cells from Afferent Lymph using Optiprep®

Protocol, adapted from K. Mattews.

Preparation

- Remove Optiprep® from the fridge and allow it get to room temperature (RT)
- Make up Buffer B (see below)

Solutions

- Keep all solutions at RT

Hepes buffered saline (HBS)

- 0.8% NaCl
- 10mM Hepes
- 0.45 μm filtered
- Alternatively, can use sPBS instead of HBS

Buffer A

- HBS(or sPBS)
- 1mM EDTA
- 0.2 μm filter the buffer

Buffer B

- Make fresh!
- Buffer A + 0.5% (w/v) BSA
- 0.2 μm filtered

Optiprep® solution 1(1:3) 1.078g/ml

- lml Optiprep®: 3m1 Buffer A

Optiprep® solution 2 (1:4) 1.068 g/ml

- I .8 ml Optiprep®: 8.2m1 Buffer A

The Optiprep® solutions must be well mixed after constitution. Every time, immediately prior to use they must be mixed well again.

1. Mix lymph end over end 6 times
2. Optional: Remove 10µl of lymph and count cells (l0µl lymph: l0µl trypan blue)
3. Transfer lymph to 50m1 Nunc tube. Note the volume!
4. Spin down the lymph: 1300rpm (300xg) @ 4^0C for 5 mins (RT in original protocol)
5. Remove supernatant
6. Re-suspend cells in residual supernatant and add a few mls of Buffer B. Pool cells and add Buffer B up to 45ml
7. Count cells
8. Spin down the lymph: 1300rpm (300xg) @ 4^0C for 5 mins (RT in original protocol)
9. Re-suspend cells in buffer B ~ 2 x10^7/ml or less in multiples of 2.5ml OR 10ml (i.e. if you have loads of cells). Do not exceed 2 x10^7 cells/ml
10. Optional: Count again
11. Transfer cells (2.5m1 volume or l0ml volume) to a sterile universal. N.B. 5 x10^7 is the optimal cell number
12. Add lml Optiprep® to *2.5* ml cells OR add 4rn1 Optiprep® to I0ml volume.
13. Mix by gentle swirling
14. Prepare Optiprep® solutions 1 and 2 (easier to do in universals). Remember to mix solutions well
15. Carefully overlay 4m1 (or 7.5m1) Optiprep solution 1. Use the autopipettor at the lowest setting to do this using a wide bore pipette
16. Overlay 10rnl (or 20m1) Optiprep® solution 2. Use a 25ml pipette (wide bore)
18. Overlay with I ml HBS or sPBS using a pasteutte
19. Spin at 600 x g for *25* minutes at RT with no brake: 1800rpm ***at*** RT in Jouan® centrifuge, brake set **on** 1)
20. Remove upper fraction (1 5m1; DC rich fraction) with a sterile pastette (leave interphase cells) and transfer cells to a sterile universal
21. Add 5m1 sPBS to collected cells
22. Spin at 200 x g, *5* minutes at RT: 1100rpm 5' at RT
23. Remove supenatant (carefully, as pellet may dislodge)
24. Re-suspend cells at the required concentration in the desired buffer (e.g. FACS buffer, usually 1 -2m1 and thcn count cells).

8.3 Appendix III

Isolation of Peripheral Blood Mononuclear Cells Using Histopaque®

1. Remove Histopaque® from the refrigerator and allow it to get to room temperature
2. Collect 20ml of whole blood and put it into a clean universal
3. Put and equal volume of PBS solution to the blood and mix by gently inverting the universal several times.
4. Pipette 9ml of Histopaque® into each of four universals
5. Reduce the speed of the automatic pipettor to the lowest possible and use the pippetor to overlay 10ml of the blood/PBS over the Histopaque®.
6. Spin the layered universal at 800G, 20°C for 20 minutes with no brake stop.
7. Using a Pasteur pipette, remove the white interface layer of PBMCs and put it into a clean universal.
8. Remove 10µl of this cell solution and count cells (l0µl lymph: l0µl trypan blue) and assess the viability percentage.
9. Wash the cells by adding sPBS to and centrifuging them at 250G, 4°C, for 5 minutes
10. Decant the supernatant and re-suspend the cell pellet in sPBS
11. Repeat the washing stage
12. Re-suspend the cells to desired concentration with appropriate media

8.4 Appendix IV

PROCEDURE FOR IMMUNOLOGICAL STAINING OF CELLS FOR FACS ANALYSIS (SINGLE STAINING).

Consumables/Chemicals/Materials

Previously labelled FACS tubes with corresponding written down entry of contents

Buffer A: 1% BSA in sPBS, 0.1% Na azide, 0.2μm filtered – Stored at 4°C

Procedure

1. Wash cells in *ice-cold* Buffer A
2. Spin cells at 1500 rpm for 5 min at 4°C
3. Count cells and make up to. 2 x 10^6 per ml). Cells should be >90% viable as determined by dye exclusion during the counting (e.g. trypan blue).
4. Dispense 50μl of cells into FACS tubes (in duplicate for each antibody)
5. Add 25μl of primary antibody at correct concentration.
6. Incubate at 4°C in a fridge for 15 minutes or on ice for 30 min.
7. Using a Nichipet® Stepper with a 12.5 ml syringe add 2 x 1.25 ml of ice cold buffer
8. Spin at 1500 rpm for 5 min and 4°C
9. Remove supernatant and resuspend cells in residual volume
10. Using a Nichipet® Stepper with a 12.5 ml syringe add 2 x 1.25 ml of ice cold buffer
11. Spin at 1500 rpm for 5 min at 4°C
12. Remove supernatant and resuspend cells in total volume of 50 μl of ice cold buffer A
13. Incubate cells with secondary fluorochrome conjugated antibody 10μl per 1x 10^6 cells (for PE) to be stained, for 15 minutes in the dark, in a fridge at 4°C.
14. Wash in buffer A twice as before in steps 7 to 12.
15. Re-suspend cells in 500μl of Buffer A, cap tubes and go to step 18 OR
16. If cells are to be stored re-suspend in 250μl of Buffer A and 250μl of ice-cold 4% paraformaldehyde.
17. Cap the FACS tubes, and cover with foil and store in a dark fridge at 4°C until analysis (within 7 days)
18. Take to FACS machine.

Double Indirect Fluorescence Staining for FACS Analysis

Procedure * (For step by step protocol Look at procedure in conjunction with other procedure for single staining)

1. Wash cells in Buffer Twice
2. Count cells and make up to 2 x 10^6 per ml (This will give 1 x 10^5 cells per 50µl)
3. Add 25 µl of each monoclonal Ab (Biotinylated and non-biotinylated)
4. Add 50µl of cells and mix
5. Incubate at 4°C for 30 min. up to an hour
6. Wash cells in Buffer Twice
7. Add 25µl of FITC/SA-PE (may need to be titred) and incubate at 4°C for 30 minutes to an hour
8. Wash cells in buffer Once

For storage up to a week, use 200µl of buffer and 200 µl of 4% paraformaldehyde

1% PARAFORMALYDEHYDE

a. Heat 50ml of PBS to 60°C

b. Add 1g of paraformaldehyde

c. Add 1M NaOH, slowly titrating until paraformaldehyde dissolves

d. Make up to 100ml and cool under running cold tap water flow

e. Adjust pH to 7

f. *Label and Date* then store at 4°C (May be used up to SEVEN DAYS TO THE DATE OF FACS ANALYSIS INCLUSIVE)

N.B. For 4% paraformaldehye, make appropriate adjustments to the amount added to the 50 ml of PBS.

8.5 Appendix V

Ovine Paratuberculosis Cases

ID	Breed	Age (years)	Sex	JD clinical Type
SH.062	Blackface	5	F	Multibacillary
SH.081	Blackface	3	F	Multibacillary
SH.029	Blackface X Bleu du Maine	4.5	F	Multibacillary
SH.100	Blackface	3	F	Multibacillary
SH.101	Blackface	2	F	Multibacillary
SH.102	Blackface	4	F	Multibacillary
SH.103	Blackface	4	F	Multibacillary
SH.104	Blackface	3	F	Multibacillary
SH.030	Texel	5.5	F	Paucibacillary
SH.027	Blackface X Bleu du Maine	4.5	F	Paucibacillary
SH.032	Blackface x Bleu du Maine	2.5	F	Paucibacillary
SH.033	Lleyn X Roussin	3.5	F	Paucibacillary
SH.083	Bleu du Maine	4	F	Paucibacillary
SH.085	Blackface x Bleu du Maine	6	F	Paucibacillary
SH.107	Texel	2.5	F	Paucibacillary
SH.108	Blackface	2.5	F	Paucibacillary
SH.084	Blackface X Bleu du Maine	7	F	Asymptomatic
SH.086	Blackface X Bleu du Maine	7	F	Asymptomatic
SH.106	Texel	1	F	Asymptomatic

Ovine Paratuberculosis Cases (continued)

ID	Breed	Age (years)	Sex	JD clinical Type
SH.111	Blackface	2.5	F	Asymptomatic
SH.113	Greyface	2-4	F	Asymptomatic
SH.115	Greyface	2-4	F	Asymptomatic
SH.116	Greyface	2-4	F	Asymptomatic
SH.118	Greyface	2-4	F	Asymptomatic
1	Dorset x	4.5	F	Control
2	unknown	unknown		Control
14	Dorset	unknown	F	Control
15	unknown	unknown		Control
26.1	Suffolk	3.5	F	Control
27.1	Dorset	3.5	F	Control
28	unknown	unknown		Control
A	unknown	unknown		Control
B	unknown	unknown		Control

8.6 Appendix VI

Zinc salts Fixation of Biopsy Material

N.B Make up Zinc Salts fixative fresh each time

Day 1

1. Place sections of fresh tissue (5-6 mm thick) into universals containing fresh Zinc salts Fixative (0.1M Tris base buffer with 0.05% Ca acetetae (pH 7-7.4) containing 0.5% Zn acetate and 0.5% Zn chloride).
2. Fix sections for 6-8 hours at room temperature.
3. Trim sections to 2-3mm thickness.
4. Fix sections in fresh ZSF for another 24-72 hours at room temperature. Send samples to Histology Lab at Easter Bush for paraffin embedding and sectioning.

Preparation of Zinc-salts fixative (1 litre)

Reagent	
0.1M Tris base	12.1 g
0.05% Calcium acetate	0.5g
0.5% Zinc chloride	5g
0.5% Zinc acetate	5g

Adjust pH of the Tris-Ca acetate buffer to 7-7.4 with concentrated HCl before addition of zinc salts, otherwise the zinc salts will not dissolve.

Reference: González L., Anderson I., Deane D., Summers C. and Buxton D (2001). Detection of Immune System Cells in Paraffin Wax-embedded Ovine Tissues. Journal of Comparative Pathology. Volume 125, Issue 1, Pages 41-47.

8.7 Appendix VII

Optimal percentage agarose used for different DNA sizes.

% Agarose	DNA Size (bp)
2.0 - 2.5	< 200
1.5 – 2.0	200 - 500
1.0 – 1.5	500 - 1,000
0.7 – 1.0	1,000 - 4,000

8.8 Appendix VIII

Optimized working concentration of the qPCR primers

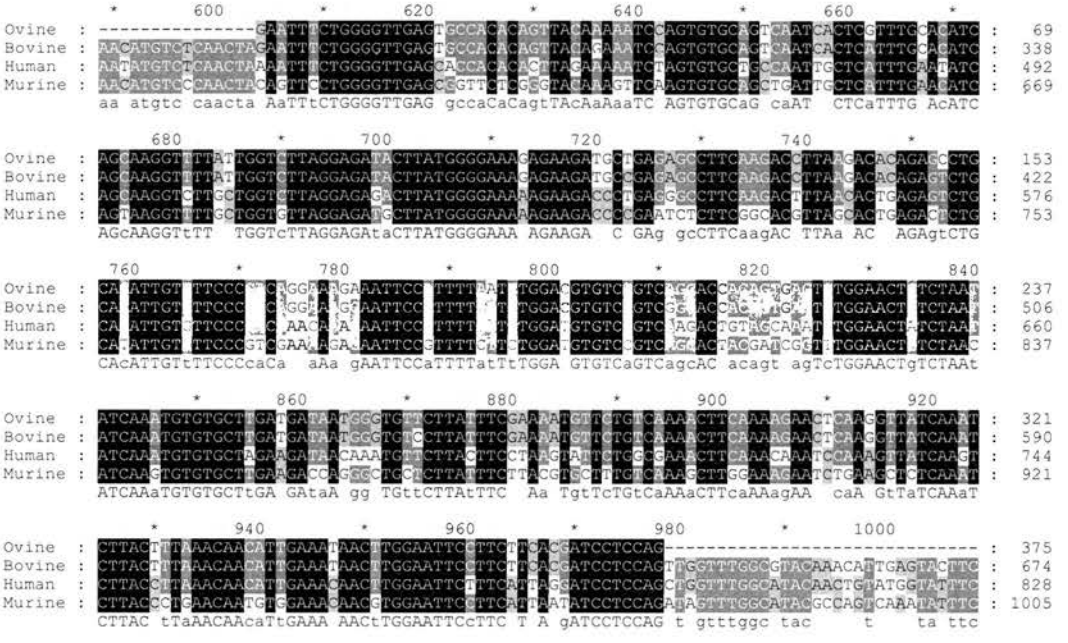
Gene Primer	Working conc		Gene primers	Working conc
TLR1	30 Mm		**TLR9**	30 Mm
TLR2	30 Mm		**TLR10**	30 Mm
TLR3	50 Mm		**MyD88**	30 Mm
TLR4	30 Mm		**CARD15**	25 Mm
TLR5	25 Mm		**CD14**	25 Mm
TLR6	25 Mm		**Dectin-1**	30 Mm
TLR7	30 Mm		**Dectin-2**	30 Mm
TLR8	30 Mm			

8.9 Appendix IX

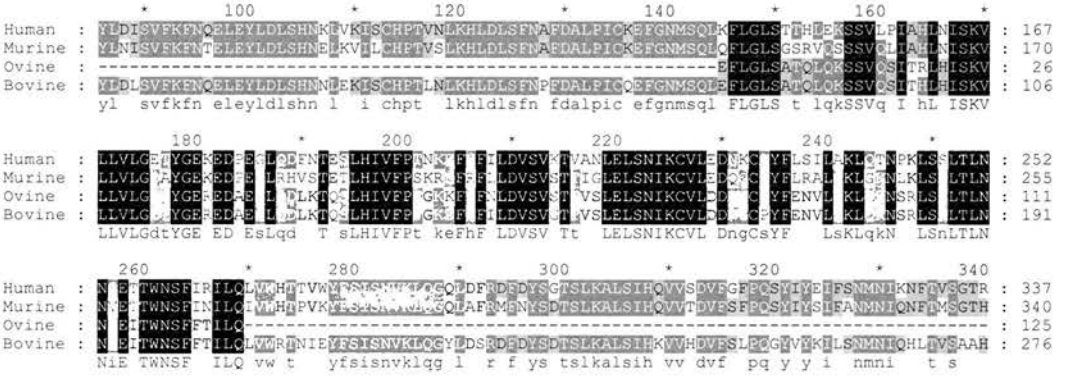
Multiple Sequence alignment of ovine nucleotide and amino acid sequences compared to bovine, human and murine sequences.

Each nucleotide sequence comparison is followed by the respective amino acid identity comparison. Identical nucleotide residues for all four sequences are shaded black, three sequences dark grey and two sequences light grey. On the consensus sequences upper case letters further represent agreement of all four sequences and lower case letter represents agreement of three sequences. For the amino acid sequences comparison, Genedoc® graphical presentation is based on similarity/conservation (conserved mode) where black (100%), dark grey (80%) and light grey (60%) amino acid conservation.

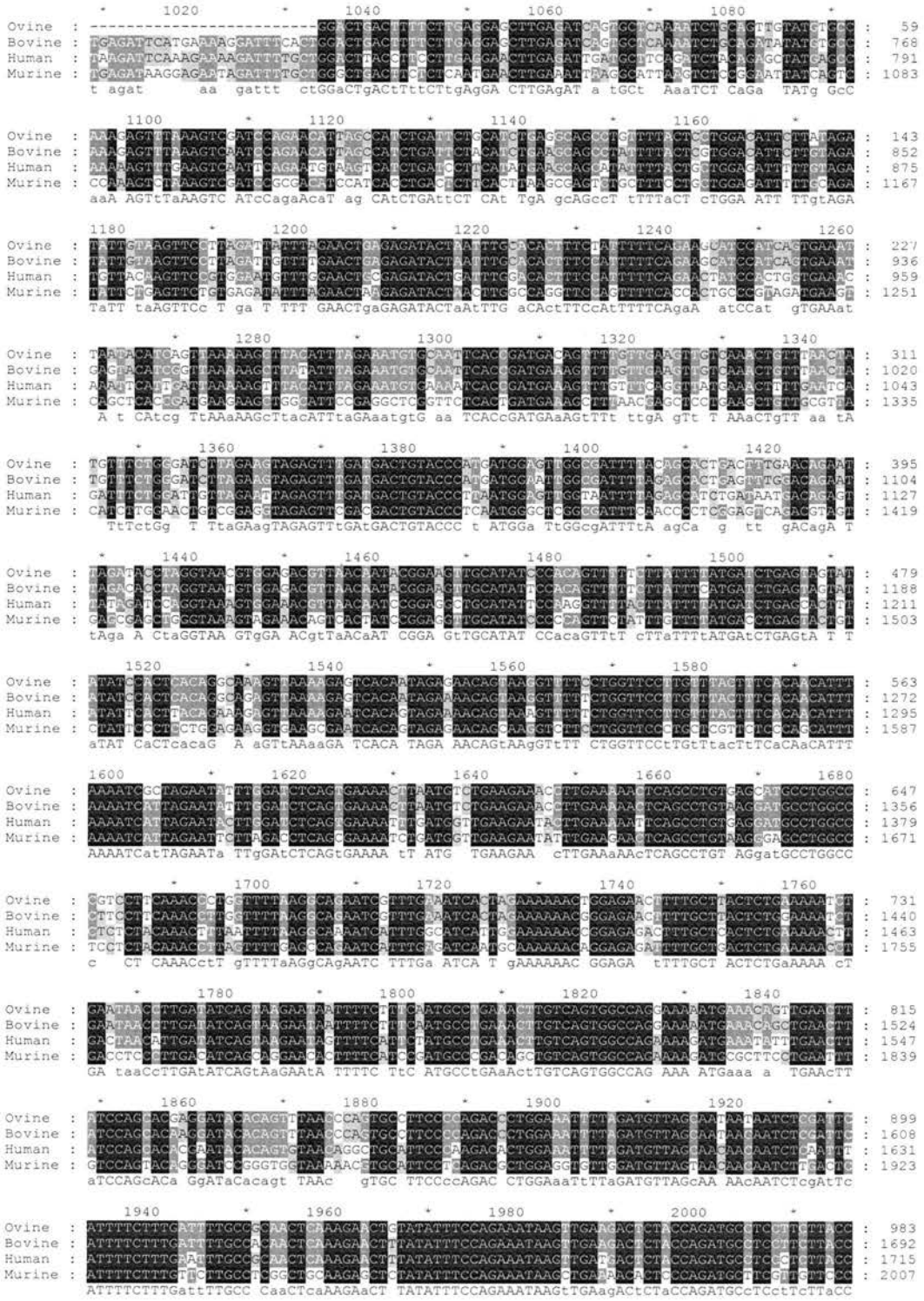
Toll-like receptor one nucleotide sequence identity comparison



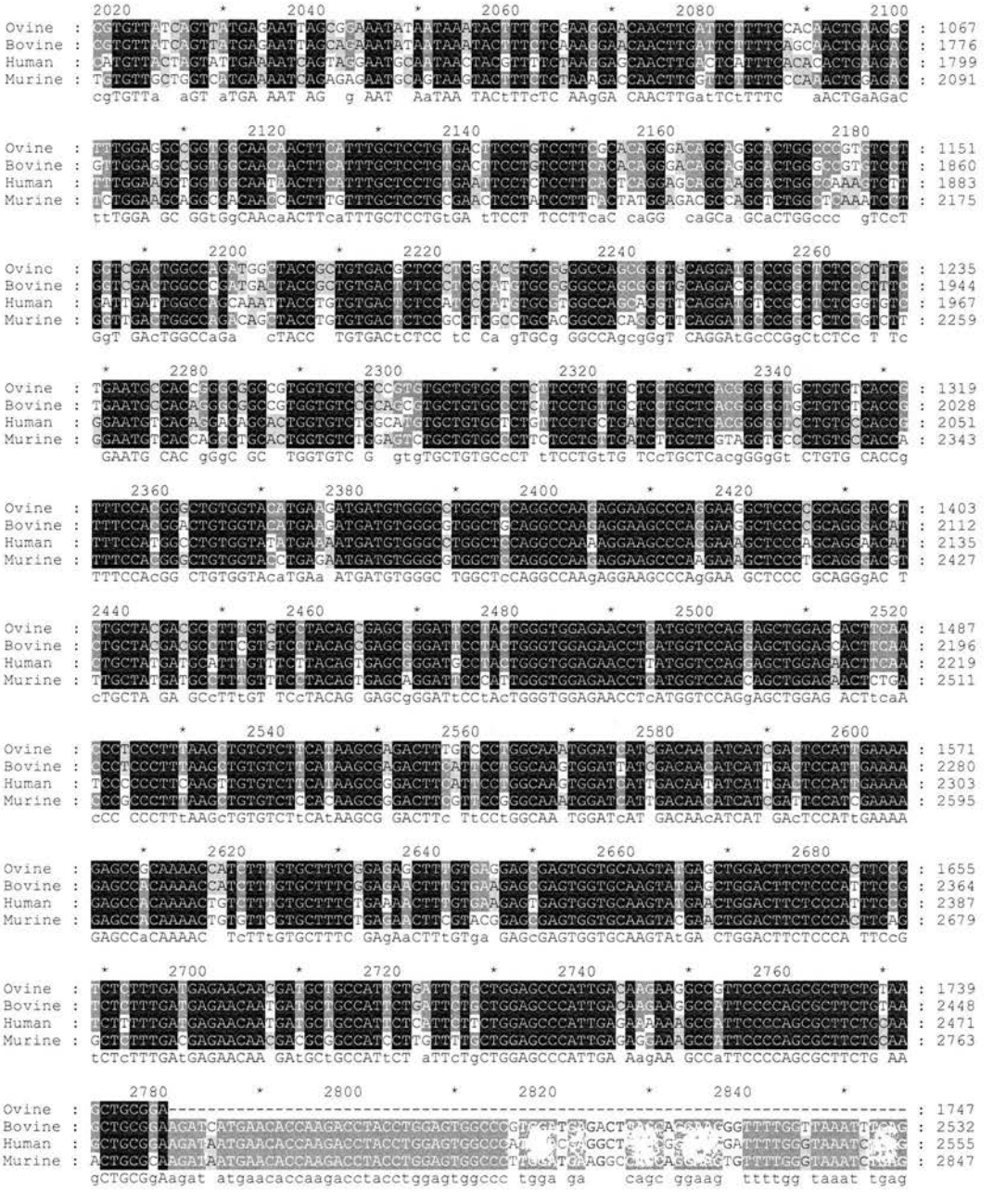
Toll-like receptor one amino acid sequence identity comparison



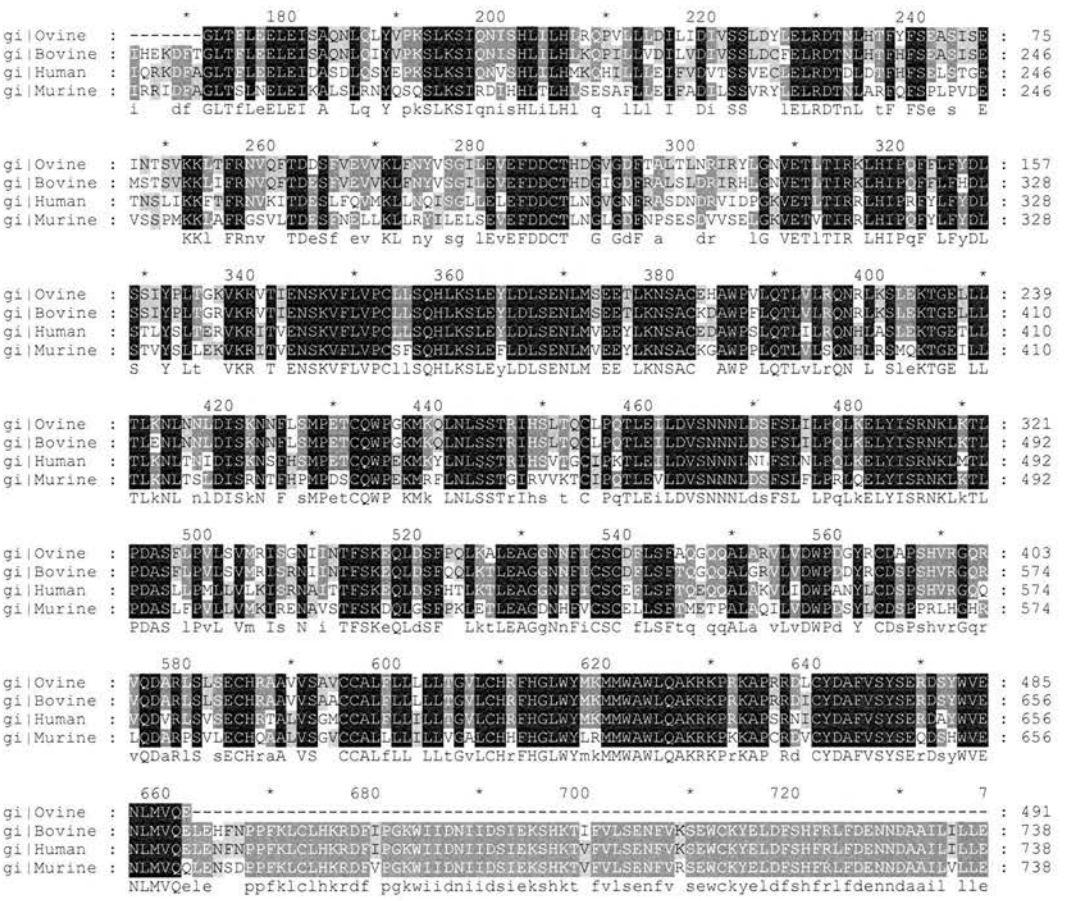
Toll-like receptor two nucleotide sequence identity comparison



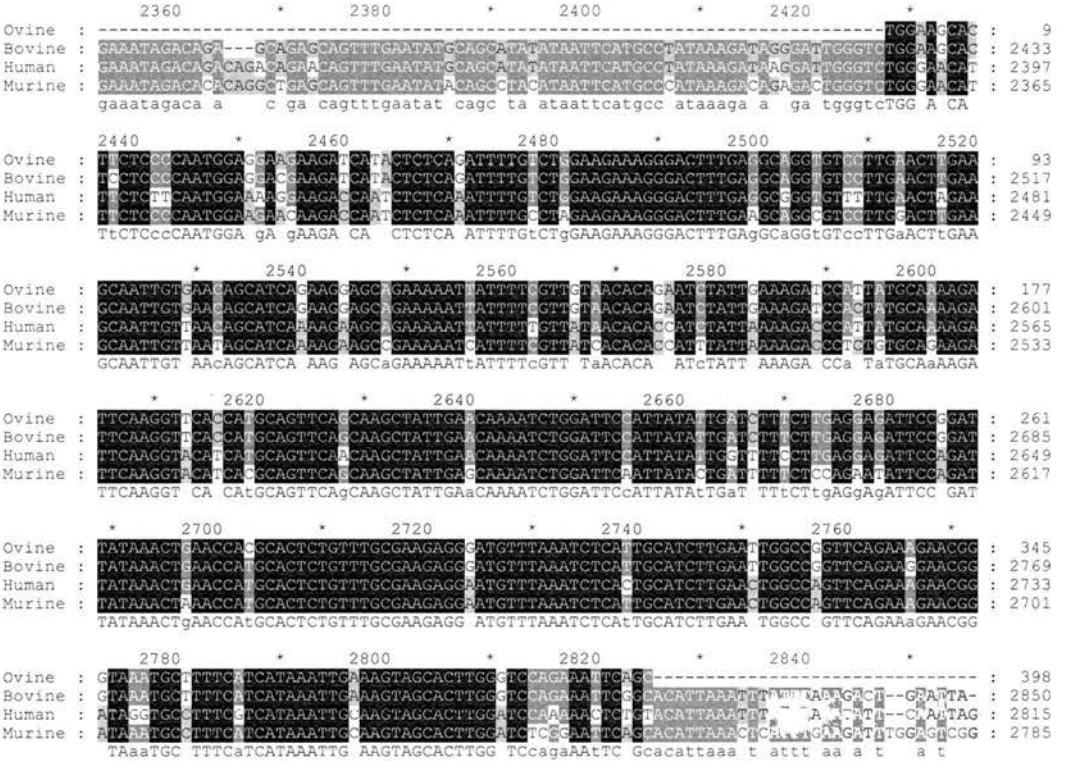
Toll-like receptor two nucleotide sequence identity comparison (continued)



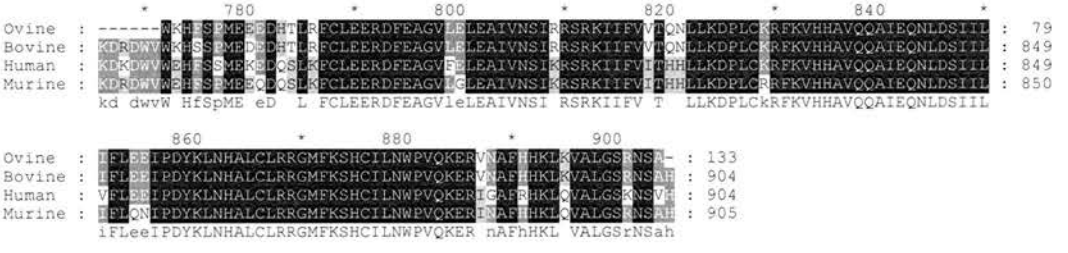
Toll-like receptor two amino acid sequence identity comparison



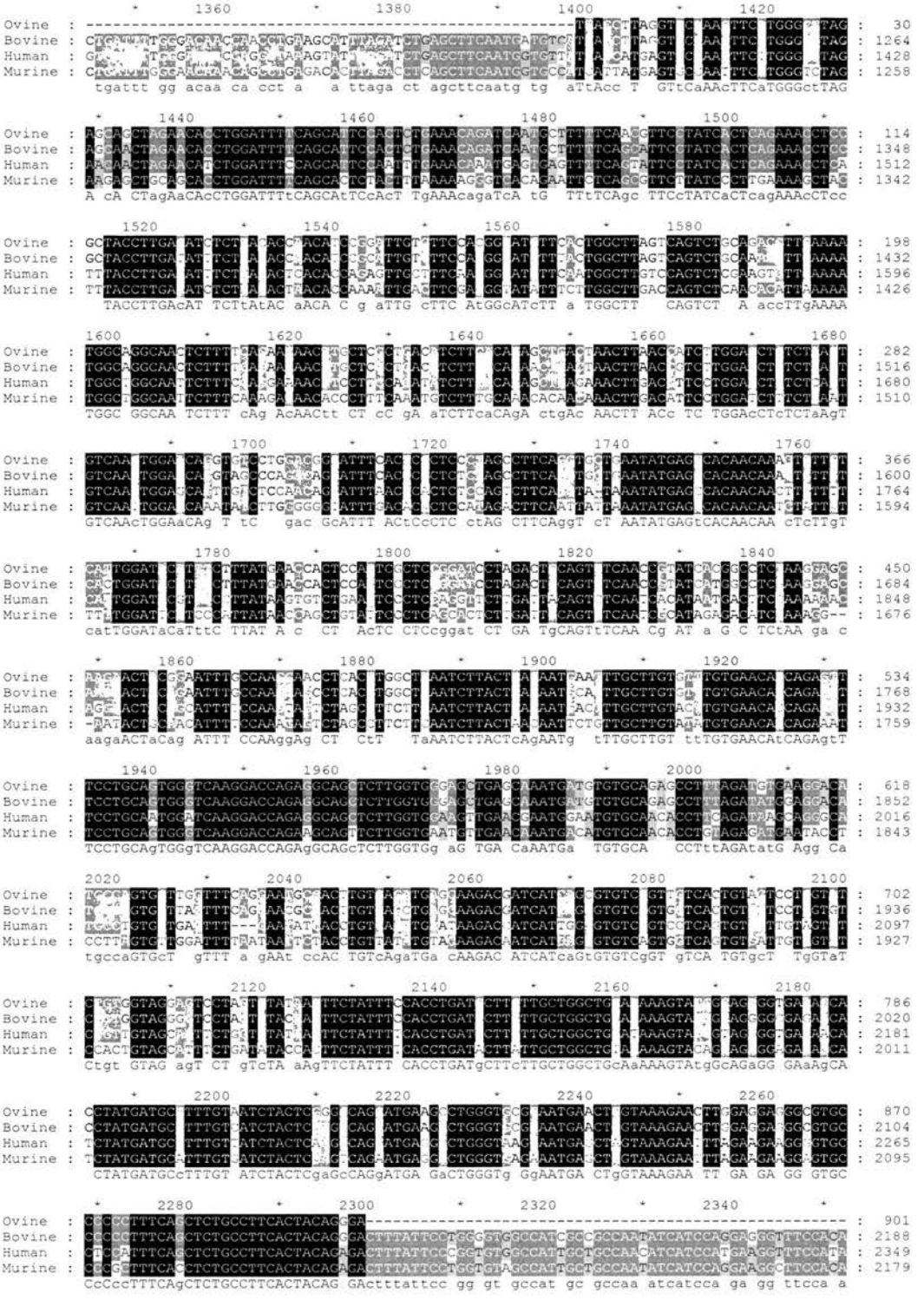
Toll-like receptor three nucleotide sequence identity comparison.



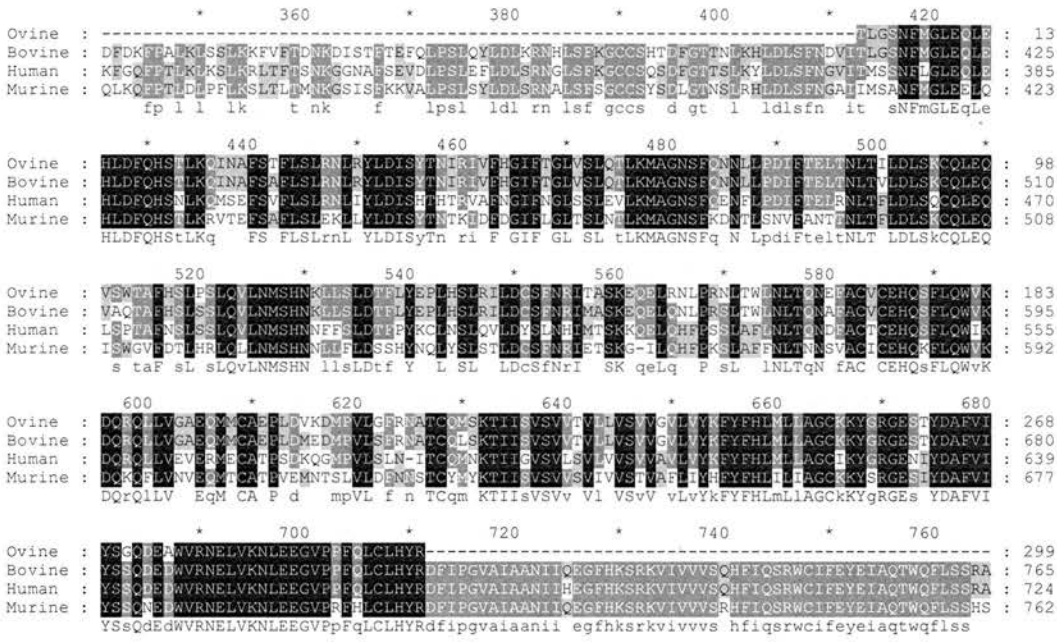
Toll-like receptor three amino acid sequence identity comparison.



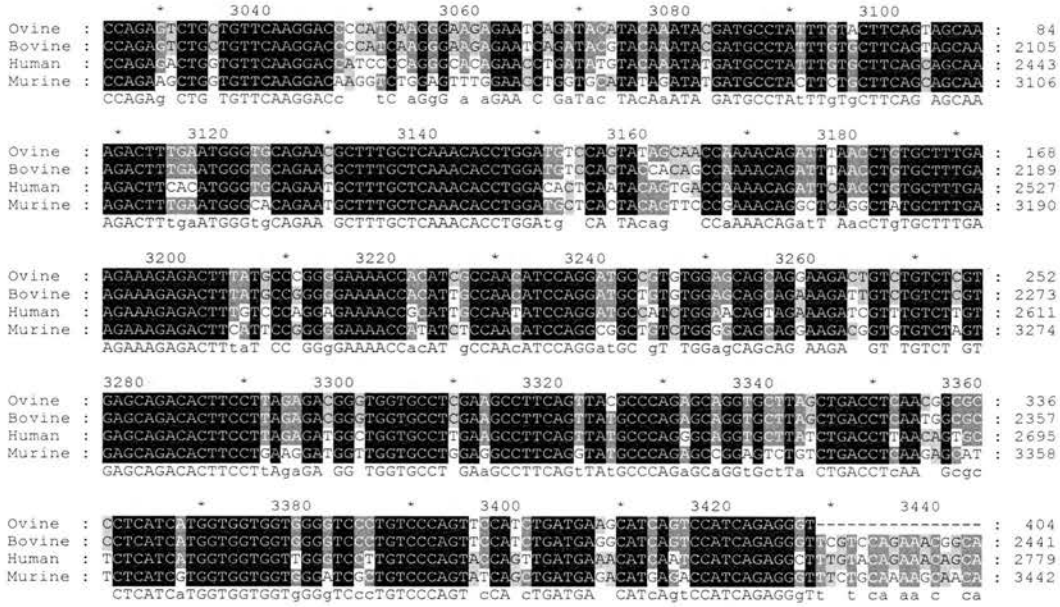
Toll-like receptor four nucleotide sequence identity comparison.



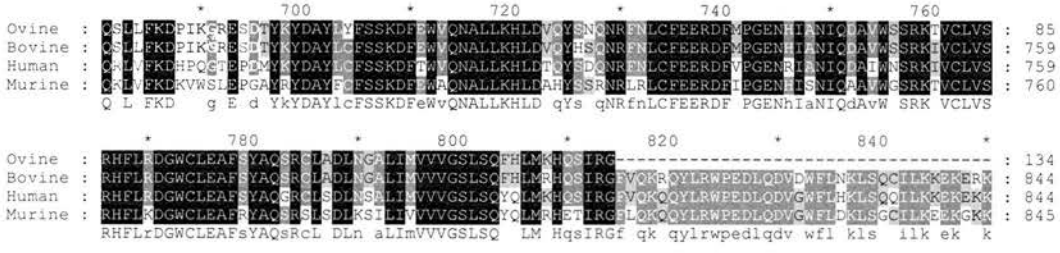
Toll-like receptor four amino acid sequence identity comparison.



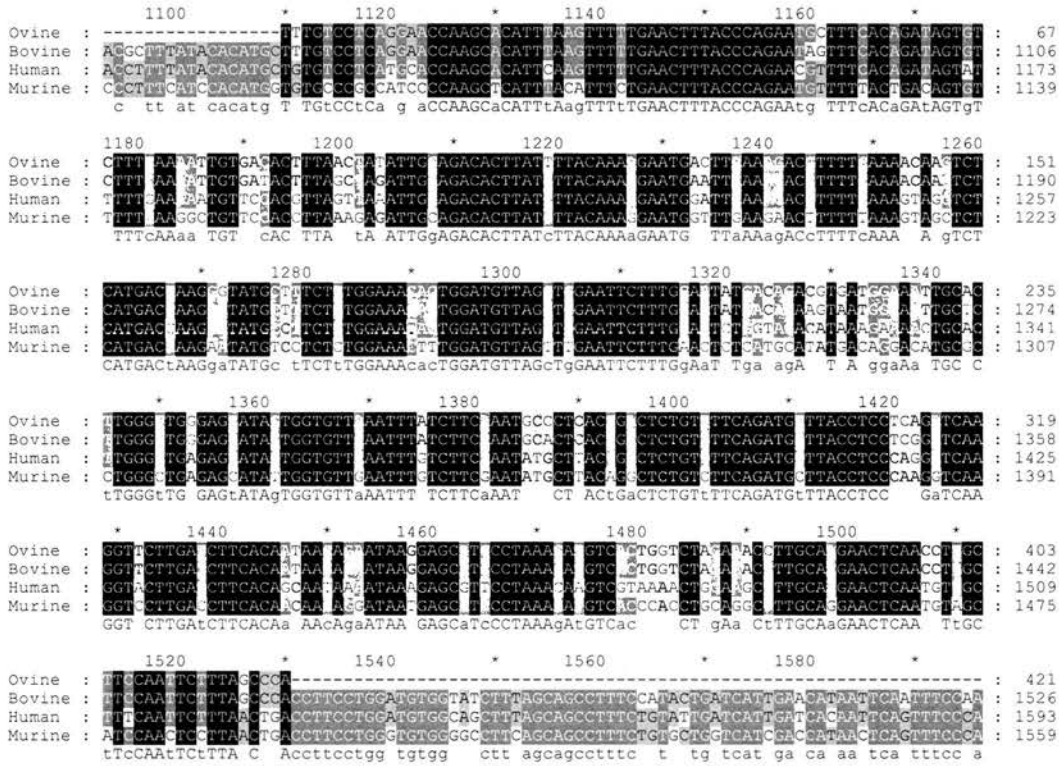
Toll-like receptor five nucleotide sequence identity comparison.



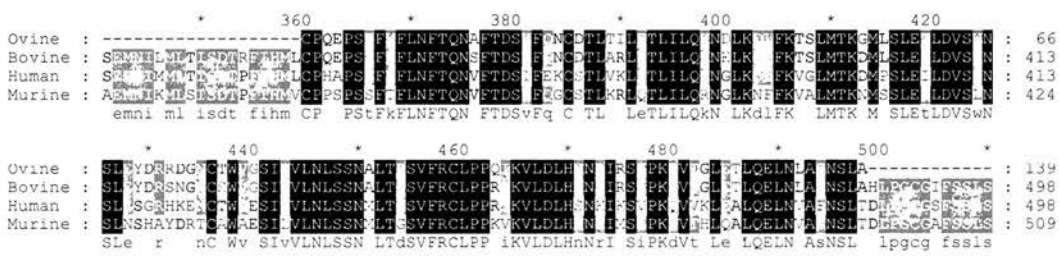
Toll-like receptor five amino acid sequence identity comparison.



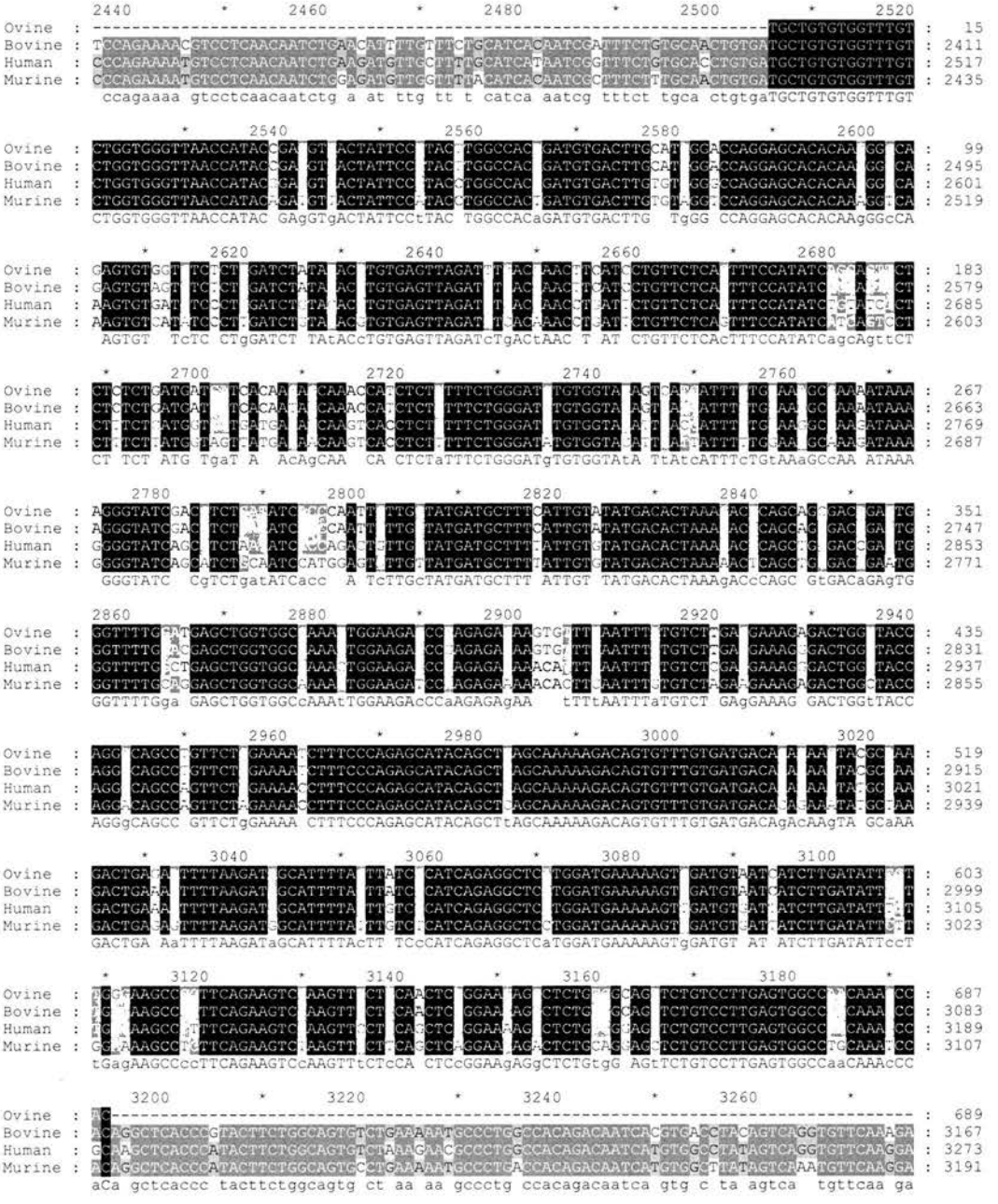
Toll-like receptor six nucleotide sequence identity comparison.



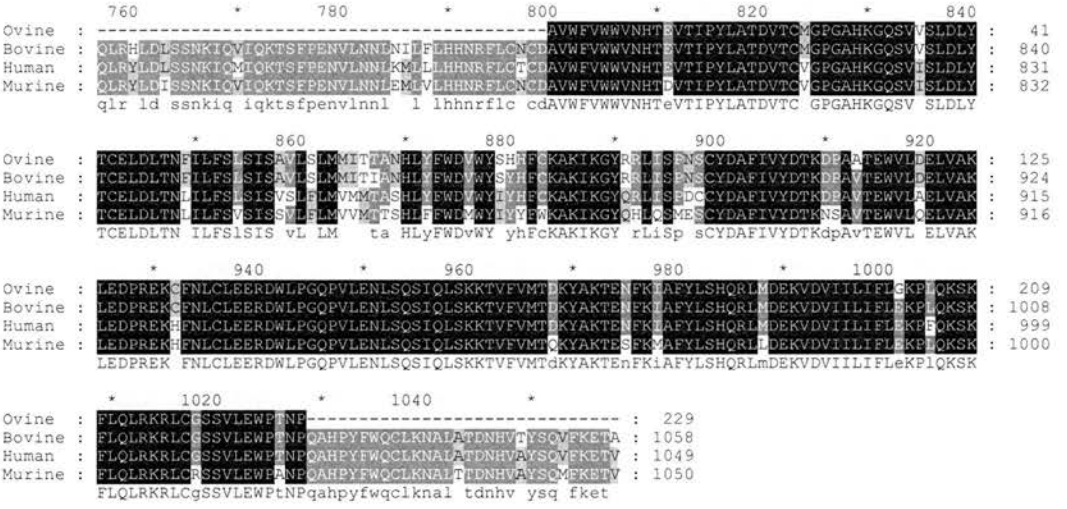
Toll-like receptor six amino acid sequence identity comparison.



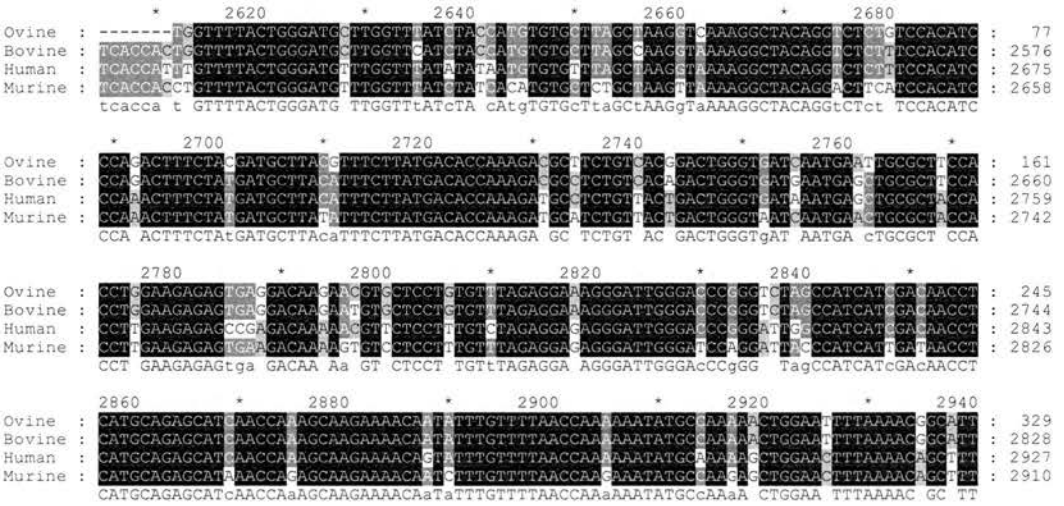
Toll-like receptor seven nucleotide sequence identity comparison.



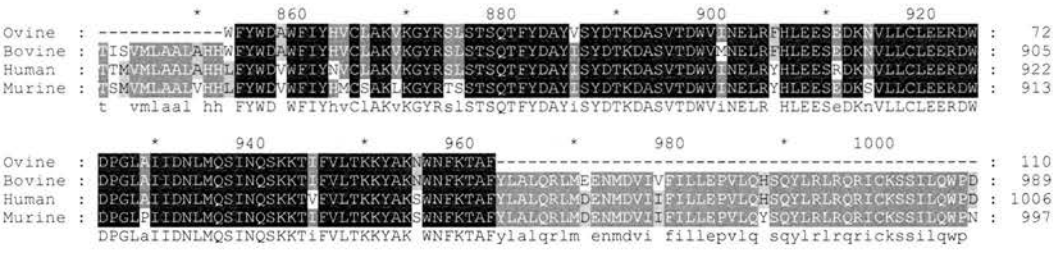
Toll-like receptor seven amino acid sequence identity comparison.



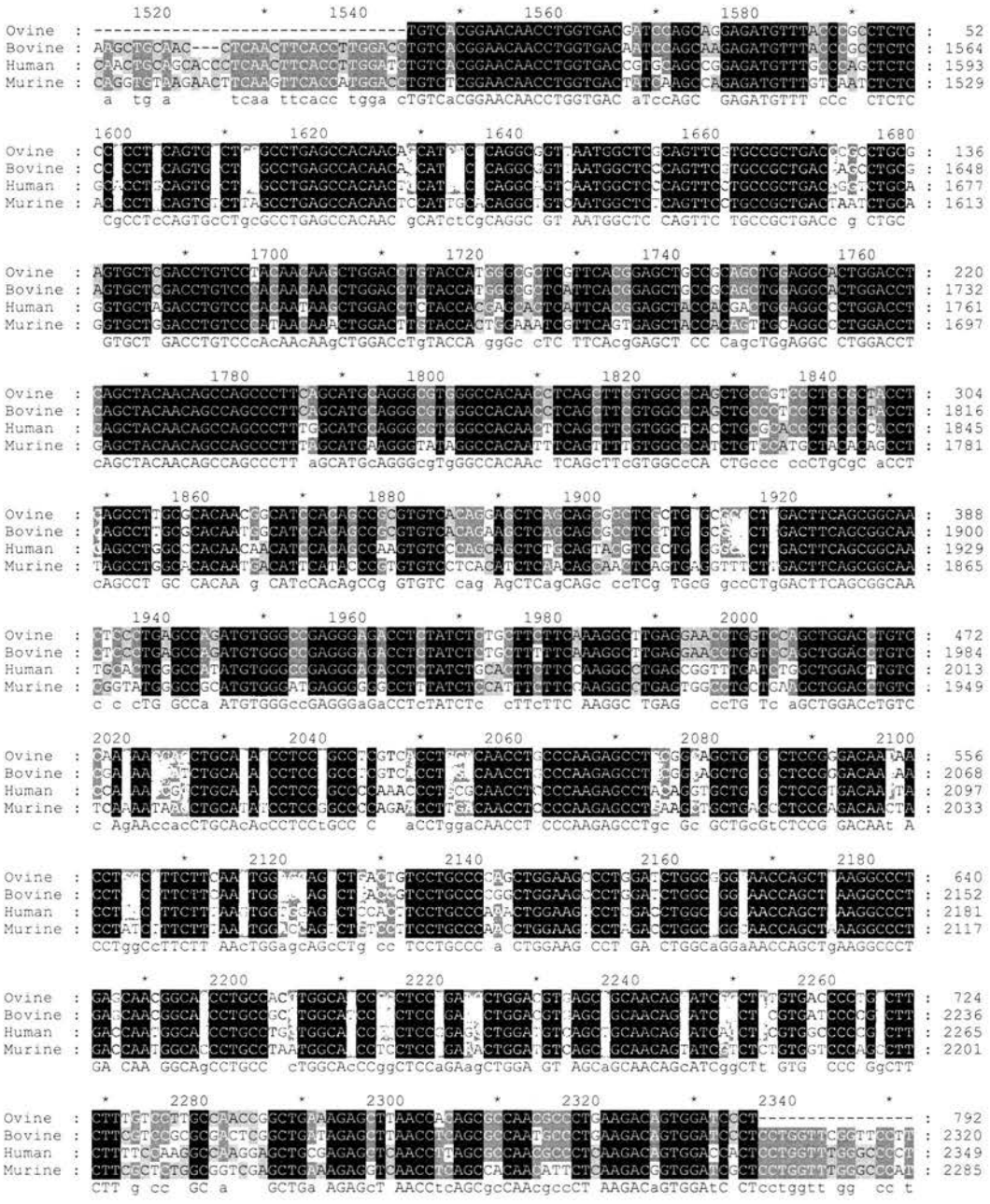
Toll-like receptor eight nucleotide sequence identity comparison.



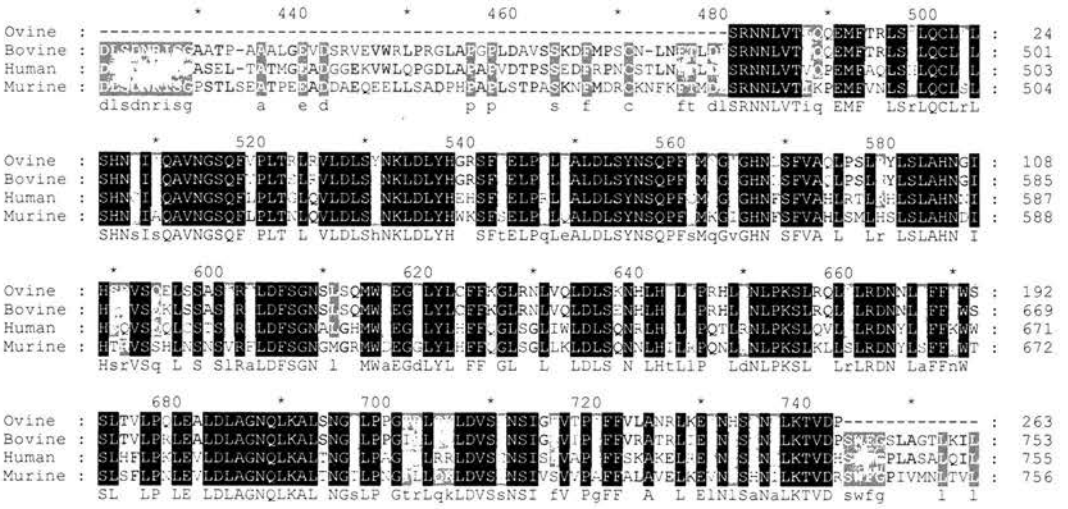
Toll-like receptor eight amino acid sequence identity comparison.



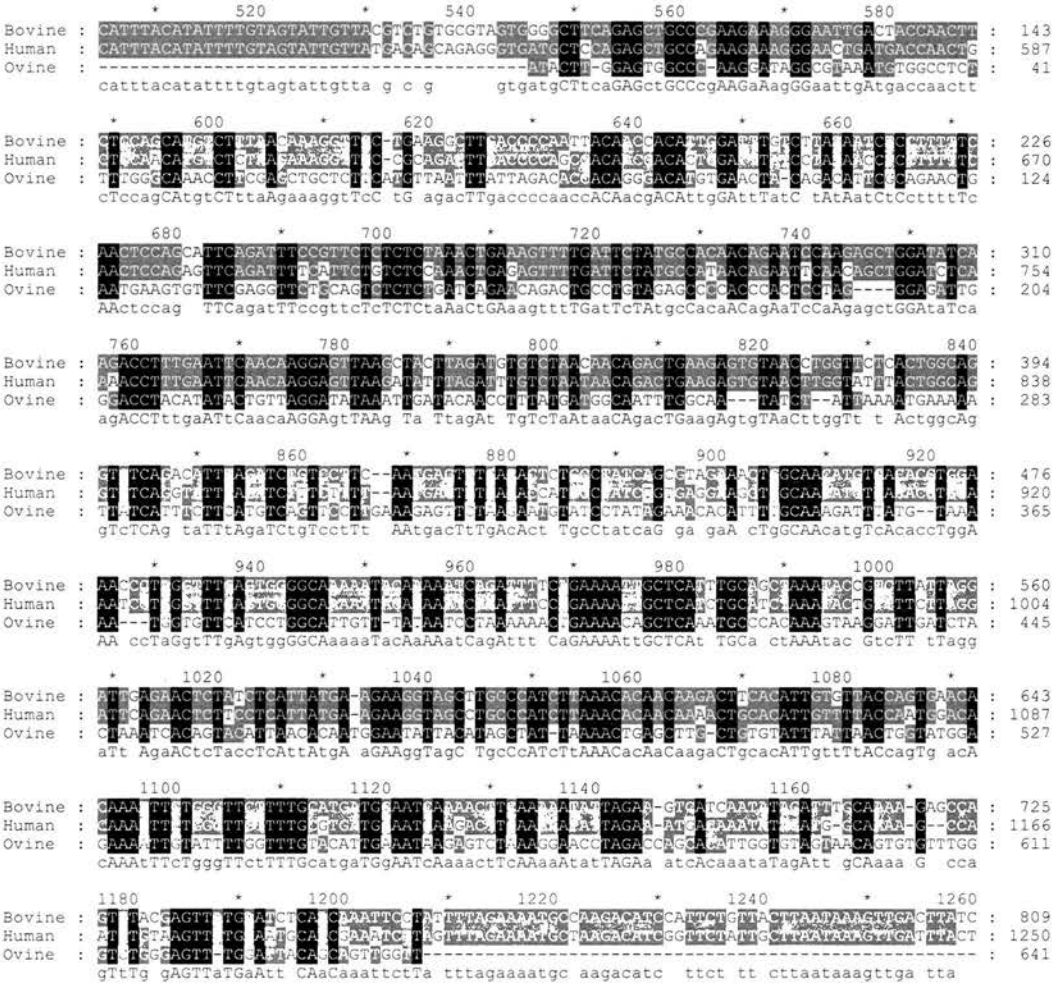
Toll-like receptor nine nucleotide sequence identity comparison.



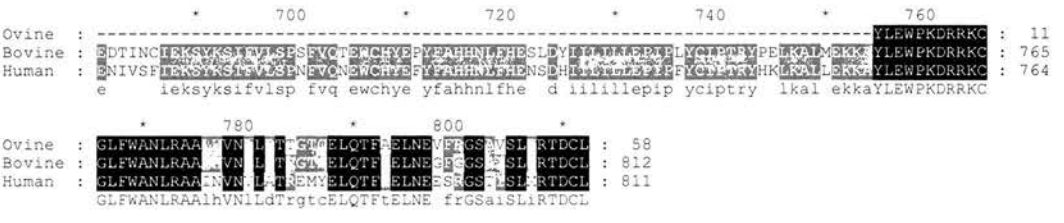
Toll-like receptor nine amino acid sequence identity comparison.



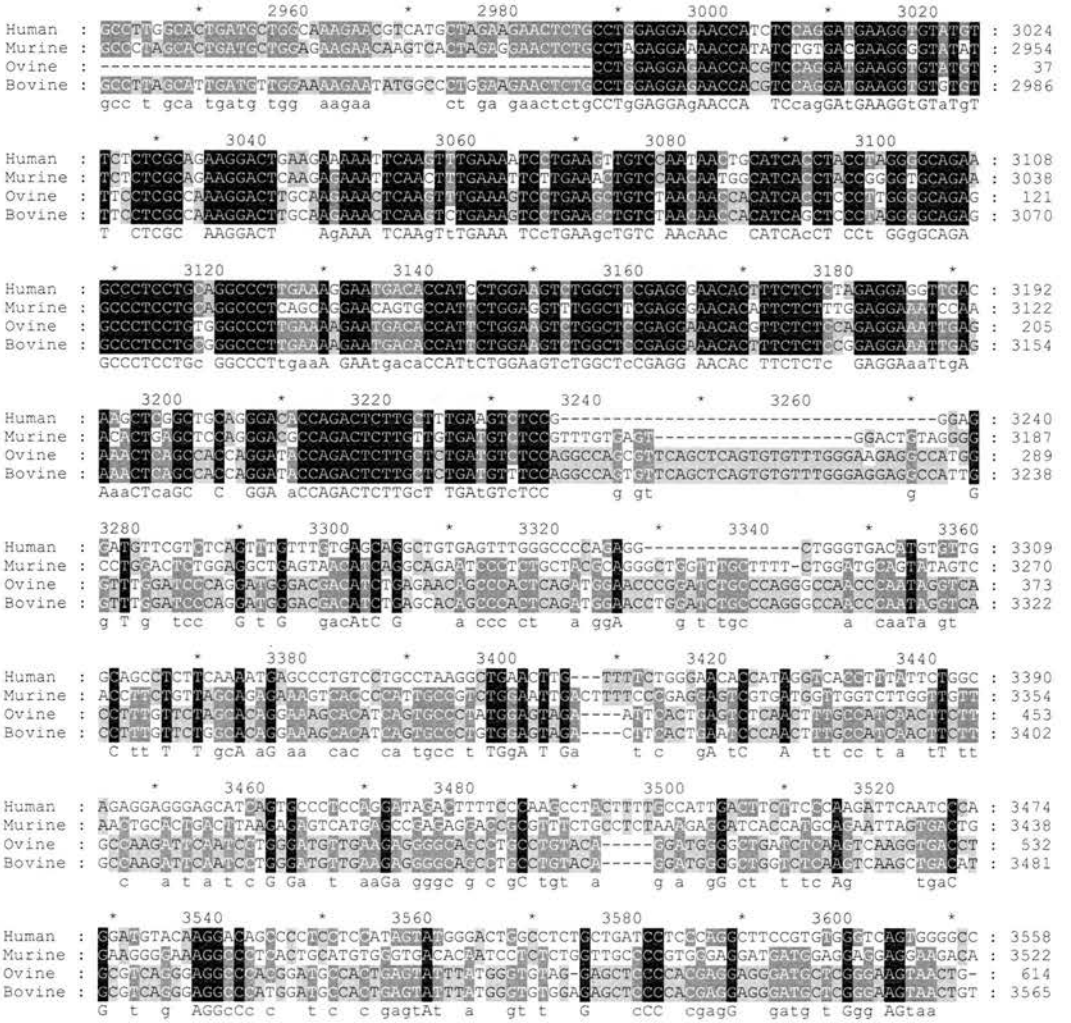
Toll-like receptor ten nucleotide sequence identity comparison.



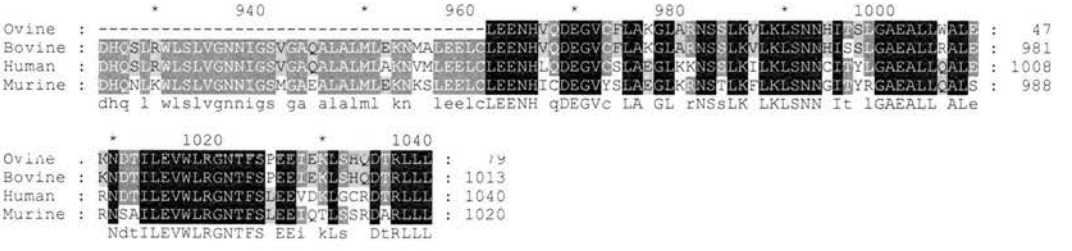
Toll-like receptor ten amino acid sequence identity comparison.



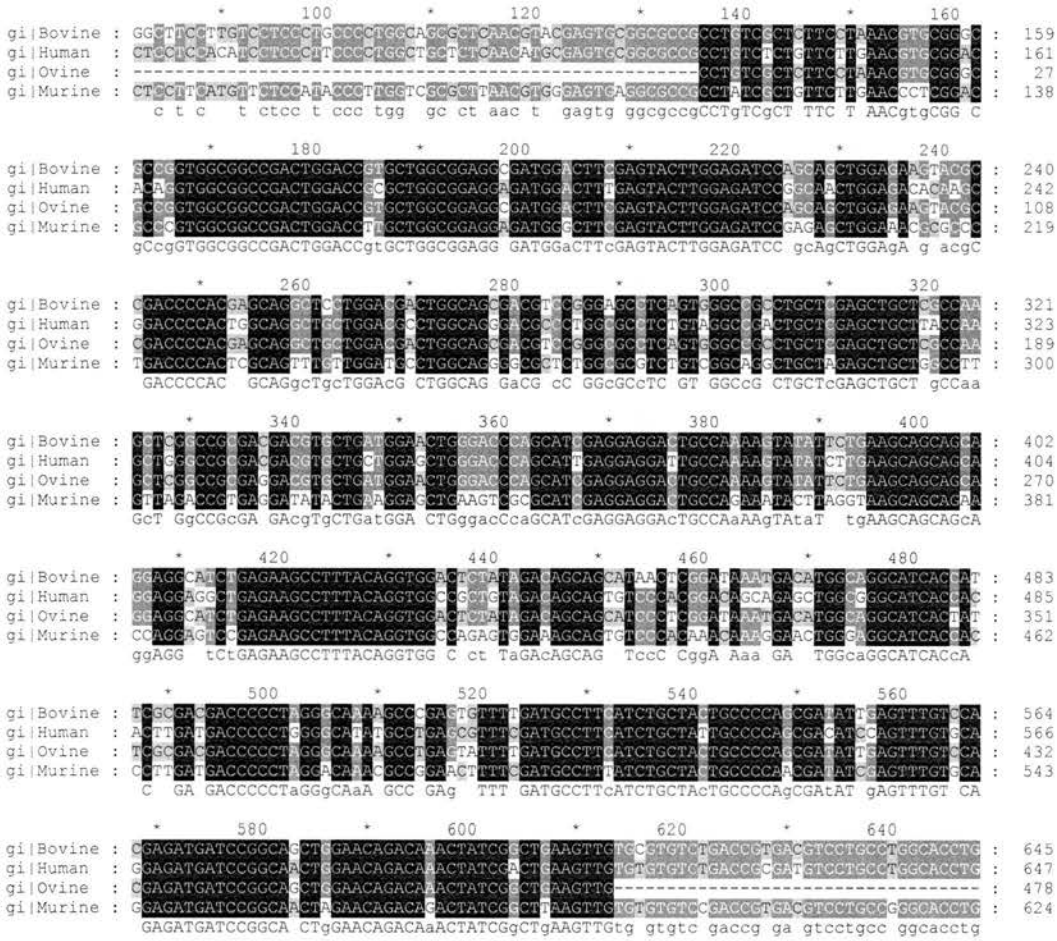
CARD15 nucleotide sequence identity comparison.



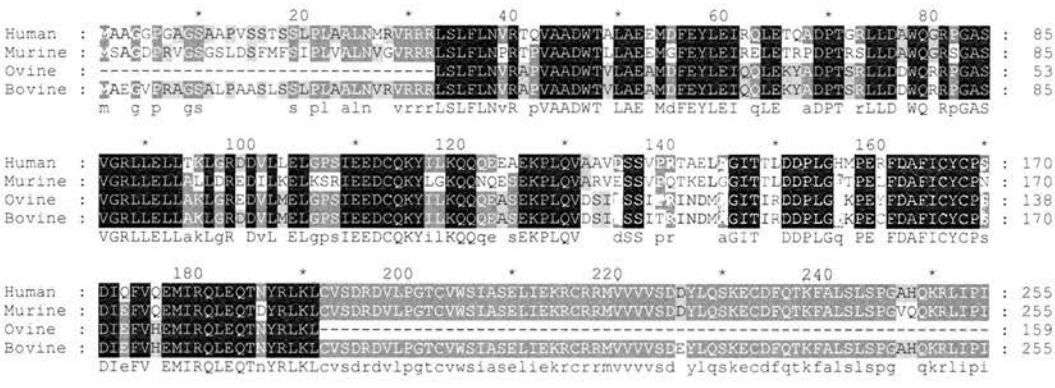
CARD15 amino acid sequence identity comparison.



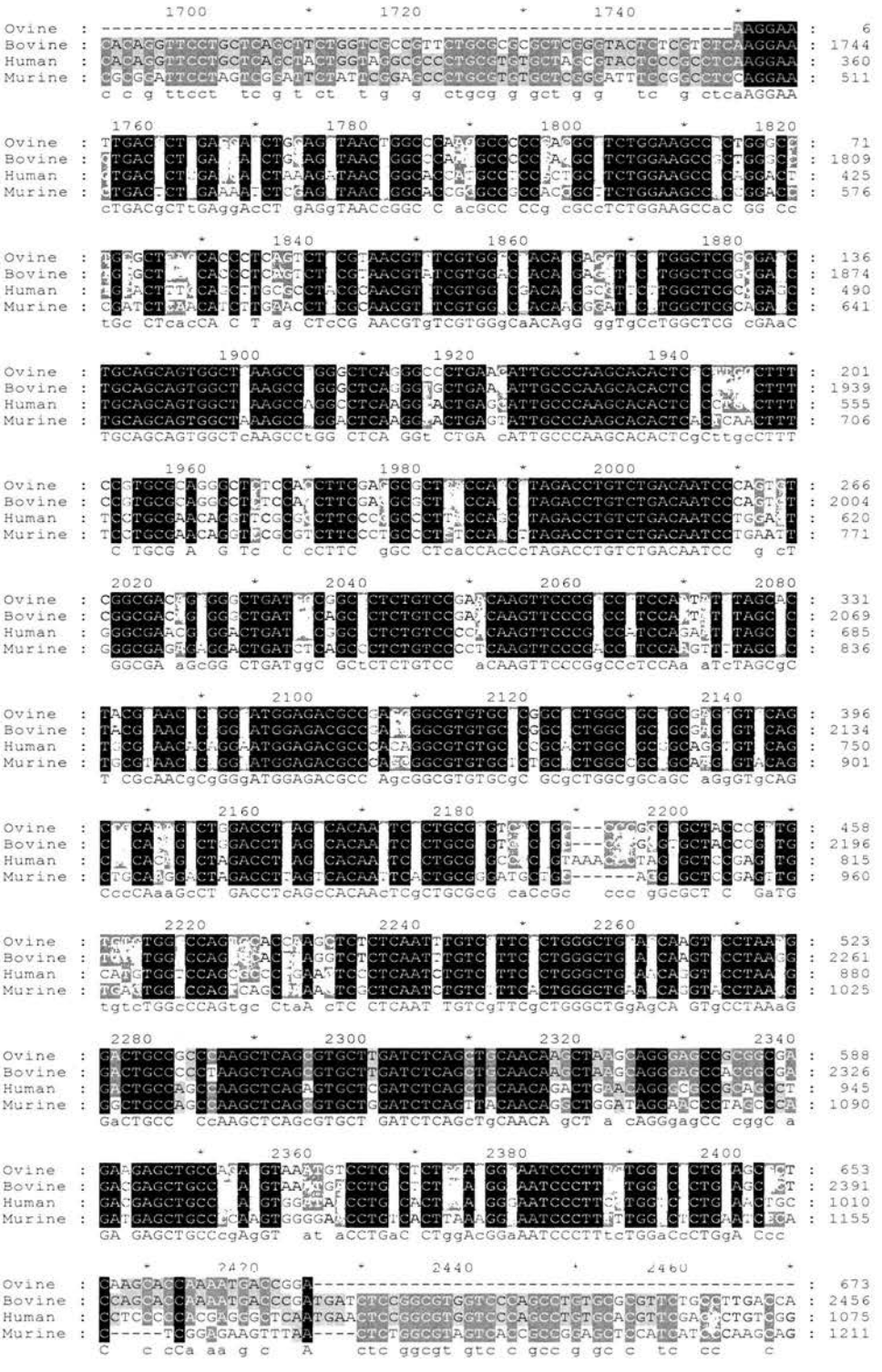
MyD88 nucleotide sequence identity comparison.



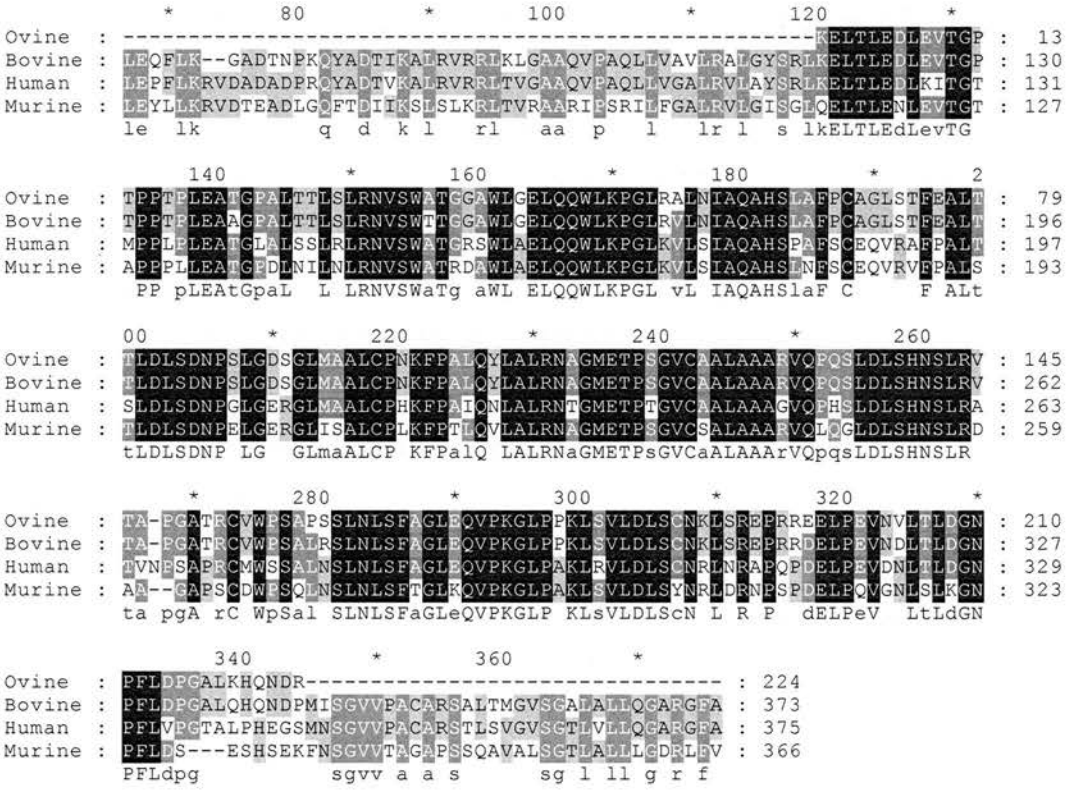
MyD88 amino acid sequence identity comparison.



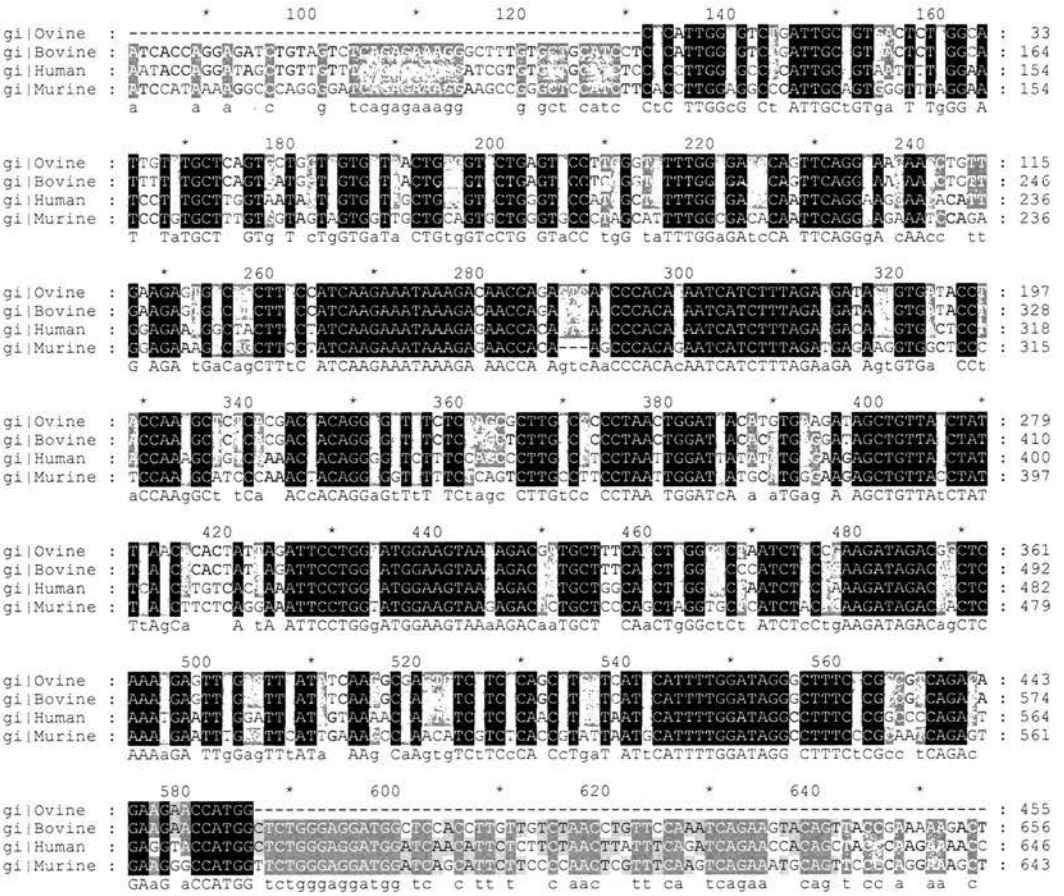
CD14 nucleotide sequence identity comparison.



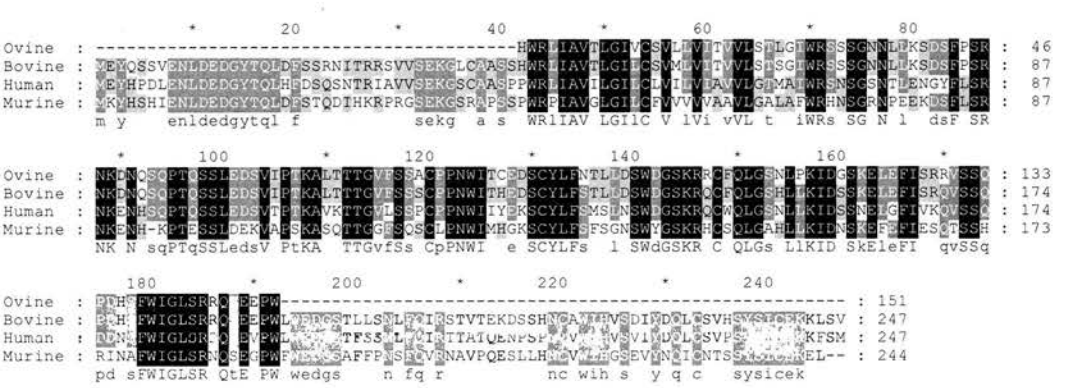
CD14 amino acid sequence identity comparison.



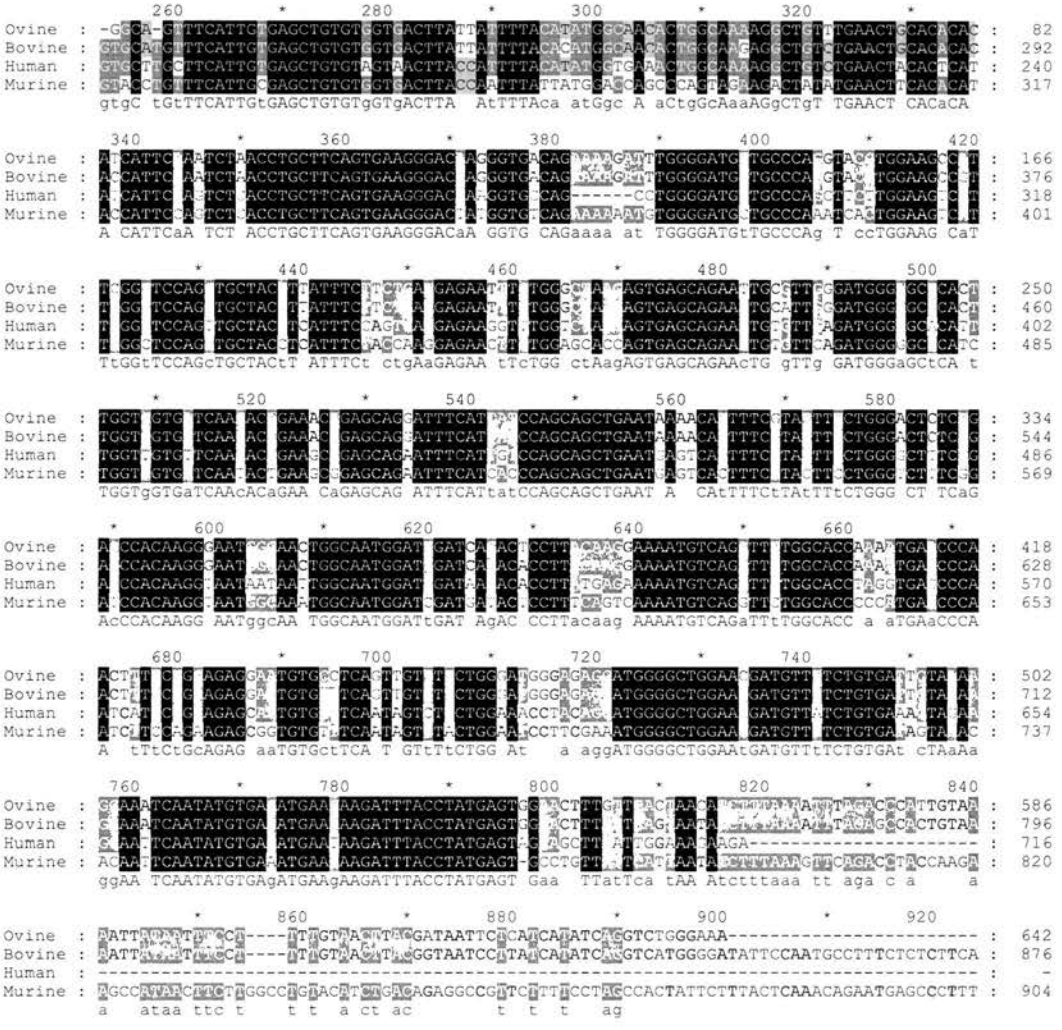
Dectin-1 nucleotide sequence identity comparison.



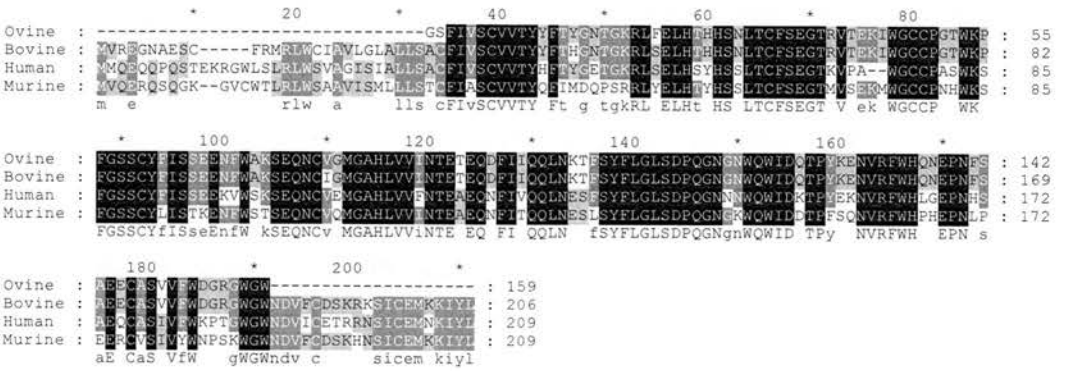
Dectin-1 amino acid sequence identity comparison.



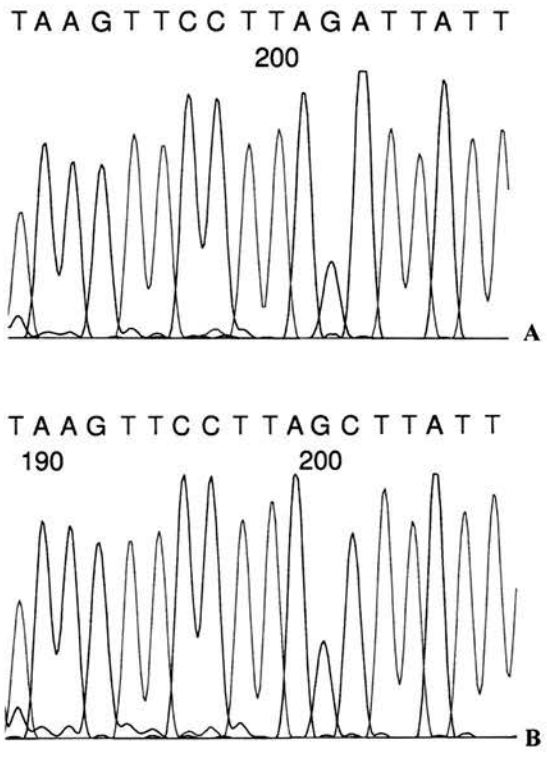
Dectin-2 nucleotide sequence identity comparison.



Dectin-2 amino acid sequence identity comparison.

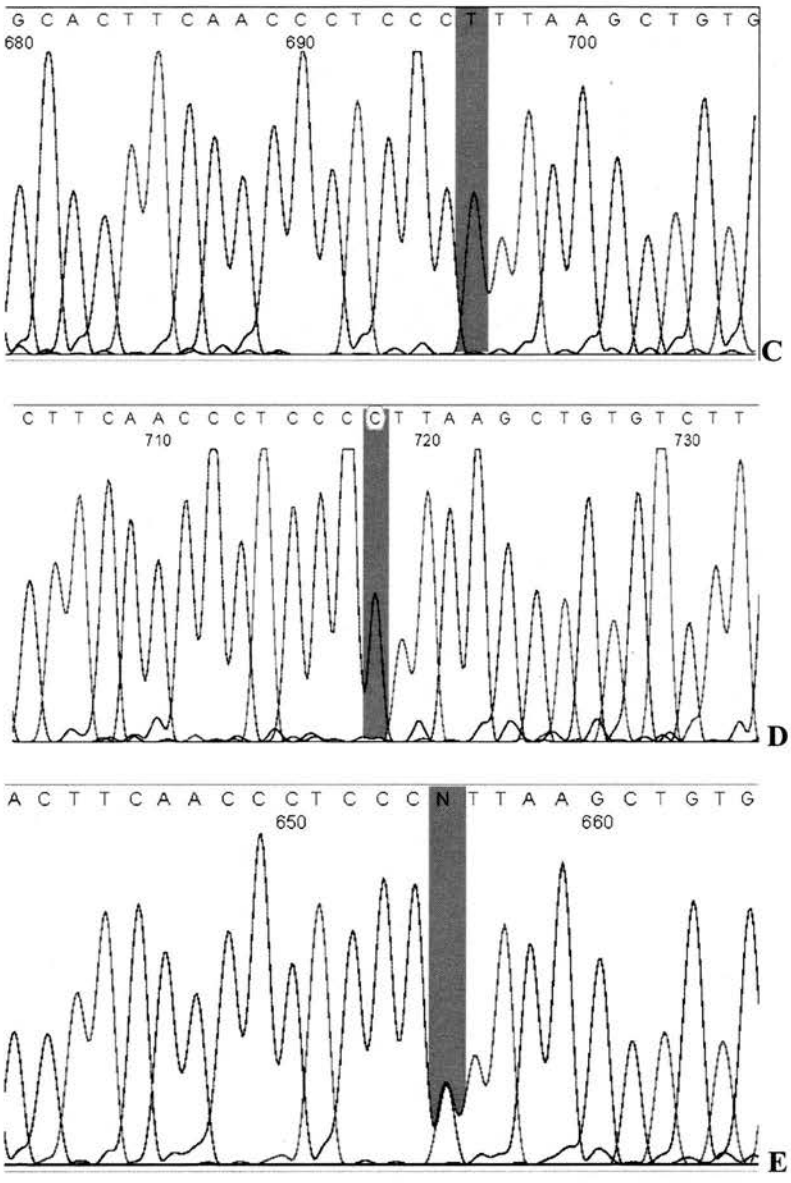


8.10 Appendix X



Sequence Chromatograph tracings of SNP182

SNP analysis of TLR2 exon2. Automatic sequencer chromatograph tracing showing the two nucleotide variations of the SNP 182 (relative to ovine TLR2 genomic sequence Accession AM117123). **A** showing the A allele and **B** showing the C allele. The amino acid changing SNPs are annotated according to the ovine TLR2 amino acid sequence (Accession CAJ65513).



Sequence Chromatograph tracings of SNP1516

SNP analysis of TLR2 exon2. The automatic sequencer chromatograph of the 1516 SNP (relative to ovine TLR2 genomic sequence Accession AM117123). **C** showing the T allele and **D** showing the C allele and **E** showing a C/T heterozygous. The amino acid changing The SNPs are annotated according to the ovine TLR2 amino acid sequence (Accession CAJ65513).

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